DNA POLYMERASE FUSIONS AND USES THEREOF

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/457,426, filed March 25, 2003. The entire teachings of the above application are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to blends of chimeric and non chimeric DNA polymerases, methods for their synthesis, and methods for their use. The DNA polymerase blends disclosed herein are useful for many recombinant DNA techniques, especially nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction (PCR) or mutagenesis.

BACKGROUND

Thermostable DNA polymerases which catalyze the template-directed polymerization of deoxyribonucleoside triphosphates (dNTPs) to form DNA, are used in a variety of in vitro DNA synthesis applications, such as DNA sequencing, DNA amplification and mutagenesis. However, thermostable DNA polymerases and their associated activities (reviewed in Abramson, 1995, in PCR Strategies, (Innis et al. ed., Academic Press, Inc.)) are not always optimal for a given application (reviewed in WO 01/61015, hereby incorporated by reference in its entirety). Because of the diversity of properties and characteristics potentially exhibited by nucleic acid polymerases generally, practitioners in the art have sought to modify, to alter, or to recombine various features of nucleic acid polymerases in an effort to develop new and useful variants of the enzyme.

One approach has been directed to the discovery and isolation of new thermophilic nucleic acid polymerases, which may possess a unique and/or improved collection of catalytic properties. As a result, thermostable nucleic acid polymerases have been isolated from a

variety of biological sources, including, but not limited to, species of the taxonomic genera, Thermus, Thermococcus, Thermotoga, Pyrococcus, and Sulfolobus.

Some of these naturally occurring thermostable DNA polymerases possess enzymatically active 3'-5'exonuclease domains, providing a natural proofreading capability and, thus, exhibiting higher fidelity than Taq DNA polymerase. However, these DNA polymerases also show slower DNA extension rates and an overall lower processivity when compared to Taq DNA polymerase, thus rendering these naturally occurring thermostable DNA polymerases less desirable for PCR, despite their higher fidelity.

In an effort to compensate for the deficiencies of individual thermostable polymerases, a second approach has been to develop multiple enzyme assemblages, combining, for example, Taq polymerase and a proofreading enzyme, such as Pfu polymerase or Vent DNA polymerase. These multiple-enzyme mixtures exhibit higher PCR efficiency and reduced error rates when compared to Taq polymerase alone (Barnes, Proc. Natl. Acad. Sci USA 91:2216-2220 (1994).).

Another approach has been to develop new and useful variants of Taq polymerase through deletion/truncation techniques. The Stoffel fragment, for example, is a 544 amino acid C-terminal truncation of Taq DNA polymerase, possessing an enzymatically active 5' 3' polymerase domain but lacking 3'-5'exonuclease and 5'-3' exonuclease activity. Other commercially available thermostable polymerase deletions include Vent (exo-) and Deep Vent (exo-) (New England Biolabs, Beverly, MA). Deletion mutations serve only to remove functional domains of a nucleic acid polymerase, however, and do not add any novel features or enzymatic properties.

Polymerase mutagenesis is yet another approach that has been attempted to develop new and useful nucleic acid polymerase variants. For example, naturally occurring DNA polymerases strongly discriminate against the incorporation of nucleotide analogues. This property contributes to the fidelity of DNA replication and repair. However, the incorporation of nucleotide analogues is useful for many DNA synthesis applications, especially DNA sequencing. Hence, a DNA polymerase that lacks associated exonucleolytic activity, either 5'-nuclease activity or 3' to 5' exonuclease activity, is preferred for DNA sequencing. In order to

generate thermostable DNA polymerases with reduced nucleotide discrimination, site-directed mutagenesis studies were initiated and resulted in the identification of mutant forms of a number of thermostable DNA polymerases with the requisite activities suitable for DNA sequencing (U.S. Pat. No. 5,466,591, incorporated herein by reference).

Yet another approach to modifying the property of a DNA polymerase is to generate DNA polymerase fusions in which one or more protein domains having the requisite activity are combined with a DNA polymerase. DNA polymerase has been fused in frame to the helix-hairpin-helix DNA binding motifs from DNA topoisomerase V and shown to increase processivity, salt resistance and thermostability of the chimeric DNA polymerase as described in Pavlov et al., 2002, Proc. Natl. Acad. Sci USA, 99:13510-13515. Fusion of the thioredoxin binding domain to T7 DNA polymerase enhances the processivity of the DNA polymerase fusion in the presence of thioredoxin as described in WO 97/29209, U.S. 5,972,603 and Bedford et al. Proc. Natl. Acad. Sci. USA 94: 479-484 (1997). Fusion of the archaeal PCNA binding domain to *Taq* DNA polymerase results in a DNA polymerase fusion that has enhanced processivity and produces higher yields of PCR amplified DNA in the presence of PCNA (Motz, M., *et al.*, J. Biol. Chem. 2002 May 3; 277 (18); 16179-88). Also, fusion of the sequence non-specific DNA binding protein Sso7d or Sac7d from *Sulfolobus sulfataricus* to a DNA polymerase, such as *Pfu* or *Taq* DNA polymerase, was shown to greatly increase the processivity of these DNA polymerases as disclosed in WO 01/92501 A1 which is hereby incorporated by reference in its entirety. Domain substitution of all or a portion of a DNA polymerase with the corresponding domain of a different DNA polymerase have also been described (U.S. 2002/0119461).

Despite these intense research efforts, there remains a need in the art to develop conditions, which are more suitable for supporting the nucleic acid synthesis, sequencing, and amplification activity of DNA polymerases.

SUMMARY OF THE INVENTION

The invention relates to methods of using a DNA polymerase fusion at high pH for DNA synthesis, DNA sequencing, cloning of a DNA synthesis product or linear or exponential PCR amplification.

One of skill in the art will understand that the DNA polymerase fusions useful according to the invention possess one or more DNA polymerase functions which are active at high pH. DNA polymerase functions are well known in the art (Ausubel et. al. Short Protocols in Molecular Biology (1995) 3rd Ed. John Wiley & Sons, Inc.; (Sambrook et al., (1989) in: Molecular Cloning, A Laboratory Manual (2nd Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N,Y.); Stratagene Catalog). Thus, the invention specifically encompasses a method of using the DNA polymerase fusions of the invention at high pH for a DNA polymerase fusion that is now known or becomes available in the art.

As used herein, "DNA polymerase function" refers to the activity of a DNA polymerase, described herein. Activities of the DNA polymerase include, but are not limited to, processivity, salt-resistance, DNA binding, strand displacement activity, polymerase activity, nucleotide binding and recognition, 3'-5' or 5'-3' exonuclease activities, proofreading, fidelity and/or decreased DNA polymerization at room temperature, as defined hereinbelow. DNA polymerase activities are well known in the art (Ausubel et. al. Short Protocols in Molecular Biology (1995) 3rd Ed. John Wiley & Sons, Inc.; (Sambrook et al., (1989) in: Molecular Cloning, A Laboratory Manual (2nd Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N,Y., see additional references incorporated by reference in their entirety herein); Stratagene Catalog). Thus, the invention specifically encompasses a method of using a DNA polymerase fusions according to the invention at high pH for a DNA polymerase activity that is now known or becomes available in the art.

DNA polymerase "function" also includes an activity of a "mutant" DNA polymerase, as defined herein. The invention encompasses but is not limited to the following activities of a "mutant" according to the invention: base analog detection activities, DNA polymerization activity, reverse transcriptase activity, processivity, salt resistance, DNA binding, strand displacement activity, nucleotide binding and recognition, 3'-5' or 5'-3' exonuclease activities, proofreading, fidelity, efficiency, specificity, thermostability and intrinsic hot start capability or decreased DNA polymerization at room temperature, decreased amplification slippage on templates with tri-nucleotide repeat stretches, decreased amplification cycles, decreased extension times, and a decrease in the amount of polymerase needed for the applications described herein. In one embodiment, the "mutant" polymerase of the invention

refers to a DNA polymerase containing one or more mutations that reduce one or more base analog detection activities of the DNA polymerase. In one embodiment, a "mutant" refers to a polymerase that has a mutation that confers an improved polymerization rate or fidelity on the polymerase. In a preferred embodiment, the "mutant" polymerase of the invention has a reduced uracil detection activity. In a preferred embodiment, the "mutant" polymerase of the invention has a reduced inosine detection activity. In another preferred embodiment, the "mutant" polymerase of the invention has a reduced uracil and inosine detection activity. In another preferred embodiment, the "mutant" polymerase of the invention has a reduced DNA polymerization activity. Any of the "mutants", for example, a mutant with reduced uracil activity, may also possess improved polymerization rate and/or fidelity, as compared to a wild-type polymerase.

The invention provides for a method for DNA synthesis at high pH, comprising: a) providing a DNA polymerase fusion; and b) contacting the fusion with a nucleic acid template, wherein the fusion permits DNA synthesis.

The invention also provides for a method for cloning of a DNA synthesis product at high pH comprising: a) providing a DNA polymerase fusion; b) contacting the fusion with a nucleic acid template, wherein the fusion permits DNA synthesis to generate a synthesized DNA product; and c) inserting the synthesized DNA product into a cloning vector.

The invention also provides for a method for sequencing DNA at high pH, comprising the steps of: (a) contacting a template DNA strand with a sequencing DNA primer; (b) contacting the DNA of step (a) with a DNA polymerase fusion, deoxyribonucleoside triphosphates, and a chain-terminating nucleotide analog; (c) incubating the mixture of step (b) under conditions sufficient to synthesize a random population of DNA molecules complementary to the first DNA molecule, wherein the synthesized DNA molecules are shorter in length than the first DNA molecule and wherein the synthesized DNA molecules comprise a terminator nucleotide at their 5′ termini; and (d) separating the synthesized DNA molecules by size so that at least a part of the nucleotide sequence of the first DNA molecule can be determined.

The invention also provides a method of linear or exponential PCR amplification at high pH for site-directed or random mutagenesis comprising the steps of: incubating a reaction mixture comprising a nucleic acid template, at least two PCR primers, and a DNA polymerase fusion under conditions which permit amplification of the nucleic acid template by the fusion to produce a mutated amplified product.

The invention also provides a method of reverse transcriptase PCR at high pH comprising the steps of incubating a reaction mixture comprising a nucleic acid template, at least one PCR primer, and a DNA polymerase fusion under conditions which permit amplification of the nucleic acid template by said fusion to produce an amplified product.

The invention provides for a composition for any one of DNA synthesis, cloning of a DNA synthesis product at high pH, sequencing DNA, linear or exponential PCR amplification for site directed or random mutagenesis, wherein the composition comprises a DNA polymerase fusion and a high pH buffer. In addition to the high pH buffer and polymerase fusion, the other components of a reaction mix may be present in the composition, e.g., template, primer, nucleotides, labels, labeled nucleotides, etc.

The invention provides for a composition for DNA synthesis, wherein the composition comprises a DNA polymerase fusion and a high pH DNA synthesis buffer. The invention contemplates a high pH DNA synthesis buffer, wherein the composition of the DNA synthesis buffer is that of a DNA synthesis buffer known in the art and described herein in the section entitled, "Applications of the Subject Invention", and wherein the DNA synthesis buffer is a "high pH" buffer, as defined herein.

The invention provides for a composition for cloning of a DNA synthesis product, wherein the composition comprises a DNA polymerase fusion and a high pH DNA cloning buffer. The invention contemplates a high pH DNA cloning buffer, wherein the composition of the DNA cloning buffer is that of a DNA cloning buffer known in the art and described herein in the section entitled, "Applications of the Subject Invention", and wherein the DNA cloning buffer is a "high pH" buffer, as defined herein.

The invention provides for a composition for sequencing DNA, wherein the composition comprises a DNA polymerase fusion and a high pH DNA sequencing buffer. The invention contemplates a high pH DNA sequencing buffer, wherein the composition of the DNA sequencing buffer is that of a DNA sequencing buffer known in the art and described herein in the section entitled, "Applications of the Subject Invention", and wherein the DNA sequencing buffer is a "high pH" buffer, as defined herein.

The invention provides for a composition for linear or exponential PCR amplification for site directed or random mutagenesis, wherein the composition comprises a DNA polymerase fusion and a high pH PCR reaction buffer. The invention contemplates a high pH PCR reaction buffer, wherein the composition of the PCR reaction buffer is that of a PCR reaction buffer known in the art and described herein in the section entitled, "Applications of the Subject Invention", and wherein the PCR reaction buffer is a "high pH" buffer, as defined herein.

In one embodiment, the methods and compositions of the invention further comprise a PCR enhancing factor and/or an additive.

In another embodiment, the DNA polymerase fusion used in the methods of the invention has reduced DNA polymerization activity.

In another embodiment, the DNA polymerase fusion comprises a Glycine to Proline substitution at amino acid position 387 (G387P) and has reduced DNA polymerization activity.

In another embodiment, the DNA polymerase fusion comprises reduced base analog detection activity.

In another embodiment, the DNA polymerase fusion comprises reduced base analog detection activity and a mutation at position V93, wherein the mutation is a Valine to Arginine substitution, a Valine to Glutamic acid substitution, a Valine to Lysine substitution, a Valine to Aspartic acid substitution or a Valine to Asparagine substitution.

In another embodiment, the DNA polymerase fusion has reduced base analog detection activity.

In another embodiment, the DNA polymerase fusion comprises reduced base analog detection activity.

In another embodiment, the DNA polymerase fusion further comprises a mutation at position V93, wherein the mutation is a Valine to Arginine substitution, a Valine to Glutamic acid substitution, a Valine to Lysine substitution, a Valine to Aspartic acid substitution or a Valine to Asparagine substitution that confers a reduced base analog detection activity phenotype to the chimeric DNA polymerase.

In another embodiment, the DNA polymerase fusion further comprises a reduced DNA polymerization activity.

In another embodiment, the DNA polymerase fusion further comprises a Glycine to Proline substitution at amino acid position 387 (G387P) that confers a reduced DNA polymerization phenotype to said chimeric DNA polymerase.

In another embodiment, the DNA polymerase fusion further comprises an Aspartate to alanine substitution at amino acid 141 (D141A) and a Glutamic acid to Alanine substitution at amino acid position 143 (D141A/E143A) that renders the chimeric DNA polymerase 3'-5' exonuclease deficient.

In another embodiment, the DNA polymerase fusion with reduced base analog detection activity further comprises an Aspartate to alanine substitution at amino acid 141 (D141A) and a Glutamic acid to Alanine substitution at amino acid position 143 (D141A/E143A) that renders the chimeric DNA polymerase 3'-5' exonuclease deficient.

In another embodiment, the DNA polymerase fusion comprises a wild type, mutant or chemically modified DNA polymerase.

In another embodiment, the DNA polymerase fusion is a proofreading polymerase.

In another embodiment, the proofreading polymerase is selected from the group consisting of Pfu, KOD, Tgo, Vent and DeepVent.

In another embodiment, the DNA polymerase fusion further comprises a polypeptide with an increase in an activity selected from the group consisting of: processivity, proofreading, fidelity, DNA binding activity, strand displacement activity, polymerase activity, nucleotide binding and recognition, efficiency, template length amplification capability, GC-rich target amplification efficiency, specificity, thermostability, intrinsic hot start capability, or salt resistance.

In another embodiment, the DNA polymerase fusion further comprises a polypeptide with a reduced activity selected from the group consisting of: DNA polymerase activity at room temperature, amplification slippage on templates with tri-nucleotide repeat stretches, extension time in a PCR reaction or amplification cycles in a PCR reaction.

In another embodiment, the DNA polymerase fusion consists of a protein domain selected from the group of : thioredoxin processivity factor binding domain of bacteriophage T7, archaeal PCNA binding domain, PCNA, the helix-hairpin-helix DNA binding motifs from DNA topoisomerase V or the DNA binding protein Sso7d or Sac7d.

The invention also provides for a kit for performing at high pH a method selected from the group consisting of: DNA synthesis; cloning of a DNA synthesis product; sequencing DNA; and linear or exponential PCR amplification, or any additional polymerase function encompassed herein, comprising a DNA polymerase fusion and packaging materials.

The kit of the invention may further comprise a high pH buffer, or a PCR enhancing factor and/or an additive.

DEFINITIONS

A "fusion" as defined herein, is a first amino acid sequence (protein) comprising a wild type or mutant DNA polymerase of the invention, joined to a second amino acid sequence defining a polypeptide that modulates one or more activities of the DNA polymerase including, but not limited to, processivity, salt-resistance, DNA binding, strand displacement

activity, polymerase activity, nucleotide binding and recognition, 3'-5' or 5'-3' exonuclease activities, proofreading, fidelity and/or decreased DNA polymerization at room temperature, wherein the first and second amino acids are not found in the same relationship in nature. A "fusion" according to the invention contains two or more amino acid sequences (for example a sequence encoding a wild type or mutant DNA polymerase and a polypeptide that increases processivity and/or salt resistance) from unrelated proteins, joined to form a new functional protein . In one embodiment a "fusion" according to the invention comprises a first amino acid sequence derived from a first polymerase species (e.g. Pfu N-terminus) and a second amino acid sequence derived from a second polymerase species (e.g. KOD C-terminus. In one embodiment, a "fusion" of the invention comprises a first amino acid sequence derived from a first polymerase and a second amino acid sequence derived from a polypeptide that is not a polymerase. In one embodiment, the amino acid sequence derived from a polypeptide that is not a polymerase is not enzymatically active.

As used herein, "enzymatically active" means catalyzing a specific enzymatic reaction.

A fusion of the invention may present a foreign polypeptide which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. The invention encompasses fusions wherein the polypeptide that increases processivity and/or salt resistance is joined N-terminally or C-terminally to, or is inserted at any internal position of a wild-type DNA polymerase or any of the mutant DNA polymerases described herein or known in the art.

In one embodiment, the fusion of the invention is a fusion DNA polymerase comprising a wild type or mutated thermostable DNA polymerase with or without 3'-5' exonuclease activity including but not limited to *Pfu* or *Taq*. The chimeric component added to the *Pfu* or *Taq* DNA polymerase is a basic or non-basic, protein or protein domain fused to the *Pfu* or *Taq* DNA polymerase at the N- or C-terminus or at any internal position such that the chimeric component and the polymerase are in a relationship that does not exist in nature. The chimeric contribution to the activity of the *Pfu* or *Taq* DNA polymerase increases or enhances processivity, DNA binding, strand displacement activity, polymerase activity,

nucleotide binding and recognition, proofreading, fidelity, and salt resistance and/or decrease DNA polymerase activity at room temperature.

A DNA polymerase fusion of the invention has a >10% increase in one or more of the following activities (using the assays described hereinbelow) as compared to a DNA polymerase that is not a fusion using a genomic and / or plasmid template: processivity, efficiency, template length amplification capability, GC-rich target amplification efficiency, specificity, thermostability; intrinsic hot start capability, proofreading activity, fidelity, DNA binding activity, strand displacement activity, nucleotide binding and recognition, and salt resistance. A DNA polymerase fusion of the invention will also have a >10% decrease as compared to a DNA polymerase that is not a fusion using a genomic and / or plasmid template in one or more of the following activities (assayed as described hereinbelow): amplification slippage on templates with tri-nucleotide repeat stretches or DNA polymerase activity at room temperature. In one embodiment, a "fusion" of the invention has an extension time in a PCR reaction that is decreased by 5 sec, preferably 15 sec and more preferably 45 sec or more, as compared to the extension time observed in the presence of a DNA polymerase that is not a fusion alone. In another embodiment, a "fusion" of the invention has a decrease in the number of amplification cycles for PCR of 1, 1-5 or 5 or more cycles, as compared to a DNA polymerase that is not a fusion alone. In another embodiment, fewer units (.001, .01, .1 or 1 or more) of a "fusion" of the invention are useful in an application of the invention as compared to a DNA polymerase that is not a fusion. In all cases where the activity of a "fusion" is compared to the activity of a DNA polymerase that is not a fusion, the DNA polymerase that is not a fusion is identical to the polymerase domain of the fusion, and only differs from the fusion by the absence of the second amino acid sequence of the fusion, as defined herein.

As used herein, a "genomic template" means a template comprising the nucleic acid material constituting the genome of a cell or an organism.

As used herein, "fused" or "joined" refers to any method known in the art for functionally connecting polypeptide domains, including without limitation recombinant fusion with or without intervening domains, intein-mediated fusion, non-covalent association, and

covalent bonding, including disulfide bonding, hydrogen bonding, electrostatic bonding, and conformational bonding.

"Domain" refers to a unit of a protein or protein complex, comprising a polypeptide subsequence, a complete polypeptide sequence, or a plurality of peptide sequences.

As used herein, the term "modulate" refers to an increase or decrease of 2 fold, preferably 5 fold, preferably 20 fold, preferably 100 fold, more preferably 500 fold or more in an activity of a DNA polymerase fusion of the invention as compared to a DNA polymerase that is not a fusion. In one embodiment, the DNA polymerase domain of the fusion comprises one or more mutations, as described herein. In this embodiment, the term "modulate" refers to an increase or decrease of 2 fold, preferably 5 fold, preferably 20 fold, preferably 100 fold, more preferably 500 fold or more in an activity of a DNA polymerase fusion of the invention as compared to a DNA polymerase that is not a fusion, wherein the DNA polymerase that is not a fusion is identical to the mutant DNA polymerase domain of the fusion but lacks the second amino acid sequence of the fusion as described herein.

A DNA polymerase fusion be used in combination with a PCR enhancing factor and/or an additive, as described herein.

As used herein, "high pH" refers to a pH that is greater than 9. A "high pH" is preferably 10 or more, for example 10, 11, 12, 13 or 14. A "high pH" includes any pH greater than 9 and up to a pH of 14, for example a pH of 9.1, 9.5, 9.8, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, or 14 is a "high pH" according to the invention.

As used herein, "polypeptide that increases processivity and/or salt resistance" refers to a domain that is a protein or a region of a protein or a protein complex, comprising a polypeptide sequence, or a plurality of peptide sequences wherein that region increases processivity, as defined herein, or increases salt resistance, as defined herein. A "polypeptide that increases processivity and/or salt resistance useful according to the invention includes but is not limited to any of the domains included in Pavlov et al., supra or WO 01/92501, for example Sso7d, Sac7d, HMF-like proteins, PCNA homologs, helix-hairpin-helix domains, for example derived from Topoisomerase V, or the thioredoxin binding domain of T7 DNA

polymerase as described in WO 97/29209, U.S. 5,972,603 and Bedford et al. Proc. Natl. Acad. Sci. USA 94: 479-484 (1997).

As used herein, "processivity" refers to the ability of a nucleic acid modifying enzyme, for example a polymerase, to remain attached to the template or substrate and perform multiple modification reactions. "Modification reactions" include but are not limited to polymerization, and exonucleolytic cleavage. "Processivity" also refers to the ability of a nucleic acid modifying enzyme, for example a polymerase, to modify relatively long (for example 0.5-1kb, 1-5kb or 5kb or more) tracts of nucleotides. "Processivity" also refers to the ability of a nucleic acid modifying enzyme, for example a DNA polymerase, to perform a sequence of polymerization steps without intervening dissociation of the enzyme from the growing DNA chains. "Processivity" can depend on the nature of the polymerase, the sequence of a DNA template, and reaction conditions, for example, salt concentration, temperature or the presence of specific proteins.

As used herein, "increased processivity" refers to an increase of 5-10%, preferably 10-50%, more preferably 50-100% or more, as compared to a wild type or mutant archael DNA polymerase that lacks a polypeptide that increases processivity and/or salt resistance as defined herein. Processivity and increased processivity can be measured according to the methods defined herein and in Pavlov et al., supra and WO 01/92501 A1. A polymerase with increased processivity that is a chimera comprising a polypeptide that increases processivity, as defined herein, is described in Pavlov et al. supra and WO 01/92501 A1.

As used herein, "increased salt resistance" refers to a polymerase that exhibits >50% activity at a salt concentration that is know to be greater than the maximum salt concentration at which the wild-type polymerase is active. The maximum salt concentration differs for each polymerase and is known in the art, or can be experimentally determined according to methods in the art. For example, Pfu is inhibited at 30mM salt (in a PCR reaction) so a Pfu enzyme with increased salt resistance would have significant activity (>50%) at salt concentrations above 30mM. A polymerase with increased salt resistance that is a fusion comprising a polypeptide that increases salt resistance, as defined herein, is described in Pavlov et al. supra and WO 01/92501 A1.

As used herein, "fidelity" refers to the accuracy of polymerization, or the ability of the polymerase to discriminate correct from incorrect substrates, (e.g., nucleotides) when synthesizing nucleic acid molecules (e.g. RNA or DNA) which are complementary to a template. The higher the fidelity of a polymerase, the less the polymerase misincorporates nucleotides in the growing strand during nucleic acid synthesis; that is, an increase or enhancement in fidelity results in a more faithful polymerase having a decreased error rate (decreased misincorporation rate).

The term "fidelity" as used herein also refers to the accuracy of DNA polymerization by a template-dependent DNA polymerase. The fidelity of a DNA polymerase is measured by the error rate (the frequency of incorporating an inaccurate nucleotide, i.e., a nucleotide that is not incorporated in a template-dependent manner). The accuracy or fidelity of DNA polymerization is maintained by both the polymerase activity and the 3'-5' exonuclease activity of a DNA polymerase. The term "high fidelity" refers to an error rate of 5×10^{-6} per base pair or lower. The fidelity or error rate of a DNA polymerase may be measured using assays known in the art. For example, the error rates of DNA polymerase mutants can be tested using the *lac*I PCR fidelity assay described in Cline, J., Braman, J.C., and Hogrefe, H.H. (96) NAR 24:3546-3551. Briefly, a 1.9kb fragment encoding the *lacIOlacZ*α target gene is amplified from pPRIAZ plasmid DNA using 2.5U DNA polymerase (i.e. amount of enzyme necessary to incorporate 25 nmoles of total dNTPs in 30 min. at 72°C) in the appropriate PCR buffer. The *lac*I-containing PCR products are then cloned into lambda GT10 arms, and the percentage of *lac*I mutants (MF, mutation frequency) is determined in a color screening assay, as described (Lundberg, K.S., Shoemaker, D.D., Adams, M.W.W., Short, J.M., Sorge, J.A., and Mathur, E.J. (1991) Gene 180:1-8). Error rates are expressed as mutation frequency per bp per duplication (MF/bp/d), where bp is the number of detectable sites in the *lacI* gene sequence (349) and d is the number of effective target doublings. For each DNA polymerase mutant, at least two independent PCR amplifications are performed.

A DNA polymerase having increased/enhanced/higher fidelity is defined as a polymerase having about 2 to about 10,000 fold, about 2 to about 5,000 fold, or about 2 to about 2,000 fold (preferably greater than about 5 fold, more preferably greater than about 10 fold, still more preferably greater than about 50 fold, still more preferably greater than about

100 fold, still more preferably greater than about 500 fold and most preferably greater than about 1000 fold) reduction in the number of misincorporated nucleotides during synthesis of any given nucleic acid molecule of a given length. For example, a mutated polymerase may misincorporate one nucleotide in the synthesis of 1000 bases compared to an unmutated polymerase misincorporating 10 nucleotides. Such a mutant polymerase would be said to have an increase of fidelity of 10 fold.

A DNA polymerase having reduced misincorporation is defined herein as either a mutated or modified DNA polymerase that has about or less than 50%, or preferably about or less than 25%, more preferably about or less than 10% and most preferably about or less than 1% of relative misincorporation compared to the corresponding unmutated, unmodified or wild type enzyme. A DNA polymerase of lower fidelity may also initiate DNA synthesis with an incorrect nucleotide incorporation (Perrion & Loeb, 1989, J. Biol. Chem. 264:2898-2905).

The fidelity or misincorporation rate of a polymerase can be determined in a sequencing reaction by other methods known in the art (Eckert & Kunkel, Nucl. Acids Res. 3739-3744(1990)). In one example, the sequence of a DNA molecule synthesized by the unmutated and mutated polymerase can be compared to the expected (known) sequence. In this way, the number of errors (misincorporation) can be determined for each enzyme and compared.

DNA binding and assays for detecting DNA binding are described in : PCT/US01/17492.

Strand displacement refers to the activity described in Hogrefe et al Methods of Enzymology (2001) 334:91-116 and Kong et al (93) J.Biol. Chem. 268:1965. Assays for measuring strand displacement activity are described in Hogrefe et al Methods of Enzymology (2001) 334:91-116 and Kong et al (93) J.Biol. Chem. 268:1965.

DNA polymerase activity at room temperature is as described in The Methods of Enzymology (2001) 334:91-116. Assays for measuring DNA polymerase activity at room temperature are described in The Methods of Enzymology (2001) 334:91-116 and in Nielson et al (1997) Strategies 10:40-43 Newsletter articles.

As used herein, "GC - rich target amplification efficiency" refers to the amplification efficiency of DNA templates that have greater than 50% GC content and are more difficult to melt during PCR. These targets frequently form secondary structure when the temperature cycles to the annealing temperature making PCR amplification difficult. "GC-rich target amplification" is assayed by performing PCR amplification on a target with greater than 50% GC content and comparing the yield of amplicon generated on a gel (see Biotechniques 2002 Apr;32(4):866, 868, 870-2, 874).

A polymerase with "intrinsic hot start capability" refers to a thermostable DNA polymerase that has very low (<25°) DNA polymerase activity at non-stringent primer annealing temperatures (≤ 45°). These polymerases and assays for their detection are described in Nielson et al (1997) Strategies 10:40-43.

"DNA slippage" or "amplification slippage on templates with tri-nucleotide repeat stretches" and assays for detection of this activity is as described in J Mol Biol 2001 Sep14;312(2):323-33, J Biol Chem 1999 Sep 24;274(39):27481-90, EMBO J 2001 May 15;20(10):2587-95, Biochemistry 1996 Jan 23;35(3):1046-53.

A DNA polymerase fusion that exhibits decreased DNA polymerase activity at room temperature preferably exhibits a shift in the activity vs. temperature profile such that reduced polymerase activity is observed at a suboptimal temperature (for example a non-specific primer annealing/extension temperature) and wild type polymerase activity is observed at stringent primer annealing/extension temperature. Such fusions are expected to exhibit improved specificity in PCR.

Methods of measuring the efficiency of a DNA polymerase are described in PCR Primer: A Laboratory Manual, 1995, CSHL Press, Cha and Thilly, pp. 37-51.

Methods of measuring template length amplification capability are described in Proc Natl. Acad. Sci USA, 2002, 99:596-601 and J. Biotechnol., 2001, 88:141-149.

Methods of measuring specificity of a DNA polymerase are described in J. Biochem. (Tokyo), 1999, 126:762-8.

Methods of measuring thermostability of a DNA polymerase are described in FEMS Microbiol. Lett, 2002, 217:89-94.

Methods of measuring nucleotide binding and recognition are described in J. Mol. Biol., 2002, 322:719-729 and Nucleic Acids Res., 2002, 30:605-13.

A "domain" useful according to the invention includes any double stranded or single stranded DNA binding domain known in the art or that becomes known in the art.

As used herein, "polymerase" refers to an enzyme that catalyzes the polymerization of nucleotide (i.e., the polymerase activity). Generally, the enzyme will initiate synthesis at the 3'-end of the primer annealed to a polynucleotide template sequence, and will proceed toward the 5' end of the template strand. A "DNA polymerase" catalyzes the polymerization of deoxynucleotides. In a preferred embodiment, the DNA polymerase according to the invention is thermostable. In another preferred embodiment, the DNA polymerase according to the invention is an archaeal DNA polymerase.

As used herein in reference to a DNA polymerase, the term DNA polymerase includes a "functional fragment thereof". A "functional fragment thereof" refers to any portion of a wild-type or mutant DNA polymerase that encompasses less than the entire amino acid sequence of the polymerase and which retains the ability, under at least one set of conditions, to catalyze the polymerization of a polynucleotide. Such a functional fragment may exist as a separate entity, or it may be a constituent of a larger polypeptide, such as a fusion protein.

The nucleic acid polymerases used in the present invention may be mesophilic or thermophilic, and are preferably thermophilic. Preferred mesophilic DNA polymerases include T7 DNA polymerase, T5 DNA polymerase, T4 DNA polymerase, Klenow fragment DNA polymerase, DNA polymerase III and the like. Preferred thermostable DNA polymerases that may be used in the methods of the invention include Taq, Tne, Tma, Pfu, Tfl, Tth, Stoffel fragment, VENT™ and DEEPVENT™ DNA polymerases, KOD, Tgo, JDF3, and mutants, variants and derivatives thereof (U.S. Pat. No. 5,436,149; U.S. Patent 4,889,818; U.S. Pat. No. 4,965,18S; U.S. Pat. No. 5,079,352; U.S. Patent 5,614,365; U.S. Pat. No. 5,374,553; U.S. Pat. No. 5,270,179; U.S. Pat. No. 5,047,342; U.S. Pat. No. 5,512,462; WO

92/06188; WO 92/06200; WO 96/10640; Barnes, W. M., Gene 112:29-35 (1992); Lawyer, F. C., et al., PCR Meth. Appl. 2:275-287 (1993); Flaman, J. -M, et al., Nuc. Acids Res. 22(15):3259- 3260 (1994)). For amplification of long nucleic acid molecules (e.g, nucleic acid molecules longer than about 3-5 Kb in length), at least two DNA polymerases (one substantially lacking 3' exonuclease activity and the other having 3' exonuclease activity) are typically used. See U.S. Pat. No. 5,436,149; U.S. Pat. No. 5,512,462; Fames, W. M., Gene 112:29-35 (1992); and copending U.S. patent application Ser. No. 09/741,664, filed Dec. 21, 2000, the disclosures of which are incorporated herein in their entireties. Examples of DNA polymerases substantially lacking in 3' exonuclease activity include, but are not limited to, Taq, Tne(exo-), Tma(exo-), Pfu(exo-), Pwo(exo-), exo-KOD and Tth DNA polymerases, and mutants, variants and derivatives thereof.

As used herein, "archaeal" DNA polymerase refers to DNA polymerases that belong to either the Family B/pol I-type group (e.g., *Pfu*, KOD, Pfx, Vent, Deep Vent, Tgo, Pwo) or the pol II group (e.g., Pyrococcus furiosus DP1/DP2 2-subunit DNA polymerase). In one embodiment, "archaeal" DNA polymerase refers to thermostable archaeal DNA polymerases (PCR-able) and include, but are not limited to, DNA polymerases isolated from Pyrococcus species (furiosus, species GB-D, woesii, abysii, horikoshii), Thermococcus species (kodakaraensis KOD1, litoralis, species 9 degrees North-7, species JDF-3, gorgonarius), Pyrodictium occultum, and Archaeoglobus fulgidus. It is estimated that suitable archaea would exhibit maximal growth temperatures of >80-85^{0}C or optimal growth temperatures of >70-80^{0}C. Appropriate PCR enzymes from the archaeal pol I DNA polymerase group are commercially available, including *Pfu* (Stratagene), KOD (Toyobo), Pfx (Life Technologies, Inc.), Vent (New England BioLabs), Deep Vent (New England BioLabs), Tgo (Roche), and Pwo (Roche). Additional archaea related to those listed above are described in the following references: Archaea: A Laboratory Manual (Robb, F.T. and Place, A.R., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1995

As used herein, "mutant" polymerase refers to a DNA polymerase, as defined herein, comprising one or more mutations that modulate, as defined herein, one or more activities of the DNA polymerase including, but not limited to, base analog detection activities, DNA polymerization activity, reverse transcriptase activity, processivity, salt resistance, DNA

binding, strand displacement activity, nucleotide binding and recognition, 3'-5' or 5'-3' exonuclease activities, proofreading, fidelity, efficiency, specificity, thermostability and intrinsic hot start capability or decreased DNA polymerization at room temperature, decreased amplification slippage on templates with tri-nucleotide repeat stretches, decreased amplification cycles, decreased extension times, and a decrease in the amount of polymerase needed for the applications described herein. In one embodiment, the "mutant" polymerase of the invention refers to a DNA polymerase containing one or more mutations that reduce one or more base analog detection activities of the DNA polymerase. In one embodiment, a "mutant" refers to a polymerase that has a mutation that confers an improved polymerization rate or fidelity on the polymerase. In a preferred embodiment, the "mutant" polymerase of the invention has a reduced uracil detection activity. In a preferred embodiment, the "mutant" polymerase of the invention has a reduced inosine detection activity. In another preferred embodiment, the "mutant" polymerase of the invention has a reduced uracil and inosine detection activity. In another preferred embodiment, the "mutant" polymerase of the invention has a reduced DNA polymerization activity. Any of the "mutants" for example a mutant with reduced uracil activity, may also possess improved polymerization rate and/or fidelity, as compared to a wild-type polymerase. A "mutant" polymerase as defined herein, includes a polymerase comprising one or more amino acid substitutions, one or more amino acid insertions, a truncation or an internal deletion. A "mutant" polymerase as defined herein includes non-fusion and fusion polymerases as defined herein.

A "mutant" polymerase as defined herein also includes a fusion polymerase wherein any of the single, double or triple mutant DNA polymerases described herein, any mutant DNA polymerase comprising an insertion, described herein, or any of the truncated, or deleted mutant DNA polymerases described herein, occur in combination with a polypeptide that modulates one or more activities of the DNA polymerase including, but not limited to, DNA polymerization activity, base analog detection activities, DNA polymerization activity, reverse transcriptase activity, processivity, salt resistance, DNA binding, strand displacement activity, nucleotide or nucleotide analog binding and recognition, sensitivity to uracil, 3'-5' or 5'-3' exonuclease activities, proofreading, fidelity efficiency, specificity, thermostability and intrinsic hot start capability or decreased DNA polymerization at room temperature, decreased amplification slippage on templates with tri-nucleotide repeat stretches, decreased

amplification cycles, decreased extension times, and a decrease in the amount of polymerase needed for the applications described herein, thereby forming a fusion, as defined herein. For example, a polypeptide that increases processivity and or salt resistance is described in WO 01/92501 A1 and Pavlov et al., 2002, Proc. Natl. Acad. Sci. USA, 99:13510-13515, herein incorporated by reference in their entirety. Other specific examples of commercially useful mutations include, but are not limited to, V93R,K,E,D in *Pfu*, which confer uracil insensitivity and D141A / E143A in *Pfu*, which eliminates 3'-5' exonuclease activity. A commercially useful truncation includes, but is not limited to the N-terminal truncation in *Taq* (Klen*Taq*) which eliminates 5'-3' exonulease activity.

As used herein, "mutation" refers to a change introduced into a parental or wild type DNA sequence that changes the amino acid sequence encoded by the DNA, including, but not limited to, substitutions, insertions, deletions or truncations. The consequences of a mutation include, but are not limited to, the creation of a new character, property, function, or trait not found in the protein encoded by the parental DNA, including, but not limited to, N terminal truncation, C terminal truncation or chemical modification. A "mutant" DNA polymerase as used herein, refers to a DNA polymerase comprising a mutation as defined herein. A "mutant" DNA polymerase of the invention can encompass a DNA polymerase "fusion" of the invention.

As used herein, a DNA polymerase with a "reduced DNA polymerization activity" is a DNA polymerase mutant comprising a DNA polymerization activity which is lower than that of the wild-type enzyme, e.g., comprising less than 10% DNA (e.g., 19.9%, 9%, 8%, 6%, 4%, 2% or less than 1%) polymerization activity of that of the wild-type enzyme or less than that of a DNA polymerase that is not a fusion. Methods used to generate and characterize *Pfu* DNA polymerases with reduced DNA polymerization activity are disclosed in the pending U.S. patent application Serial No.: 10/035,091 (Hogrefe, et al.; filed: December 21, 2001); the pending U.S. patent application Serial No.: 10/079,241 (Hogrefe, et al.; filed February 20, 2002); the pending U.S. patent application Serial No.: 10/208,508 (Hogrefe et al.; filed July 30, 2002); and the pending U.S. patent application Serial No.: 10/227,110 (Hogrefe et al.; filed August 23, 2002), the contents of which are hereby incorporated in their entirety. The invention contemplates a DNA polymerase fusion with reduced DNA polymerization activity.

As used herein, "proofreading" activity refers to 3' to 5' exonuclease activity of a DNA polymerase.

A "non-proofreading" enyzme refers to a DNA polymerase that is "3' to 5' exonuclease deficient" or "3' to 5' exo-".

As used herein, "3' to 5' exonuclease deficient" or "3' to 5' exo-" refers to an enzyme that substantially lacks the ability to remove incorporated nucleotides from the 3' end of a DNA polymer. DNA polymerase exonuclease activities, such as the 3' to 5' exonuclease activity exemplified by members of the Family B polymerases, can be lost through mutation, yielding an exonuclease-deficient polymerase. As used herein, a DNA polymerase that is deficient in 3' to 5' exonuclease activity substantially lacks 3' to 5' exonuclease activity. "Substantially lacks" encompasses a complete lack of activity, for example, 0.03%, 0.05%, 0.1%, 1%, 5%, 10%, 20% or even up to 50% of the exonuclease activity relative to the parental enzyme. Methods used to generate and characterize 3'-5' exonuclease DNA polymerases including the D141A and E143A mutations as well as other mutations that reduce or eliminate 3'-5' exonuclease activity are disclosed in the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000). Additional mutations that reduce or eliminate 3' to 5' exonuclease activity are known in the art and contemplated herein.

As used herein, "synthesis" refers to any in vitro method for making a new strand of polynucleotide or elongating existing polynucleotide (i.e., DNA or RNA) in a template dependent manner. Synthesis, according to the invention, includes amplification, which increases the number of copies of a polynucleotide template sequence with the use of a polymerase. Polynucleotide synthesis (e.g., amplification) results in the incorporation of nucleotides into a polynucleotide (i.e., a primer), thereby forming a new polynucleotide molecule complementary to the polynucleotide template. The formed polynucleotide molecule and its template can be used as templates to synthesize additional polynucleotide molecules.

"DNA synthesis", according to the invention, includes, but is not limited to, PCR, the labelling of polynucleotide (i.e., for probes and oligonucleotide primers), and polynucleotide

sequencing. The invention contemplates mutant DNA polymerases, and fusions thereof, that exhibit reduced base analog detection (for example, reduced detection of a particular base analog such as uracil or inosine or reduced detection of at least two base analogs).

As used herein, "base analogs" refer to bases that have undergone a chemical modification as a result of the elevated temperatures required for PCR reactions. In a preferred embodiment, "base analog" refers to uracil that is generated by deamination of cytosine. In another preferred embodiment, "base analog" refers to inosine that is generated by deamination of adenine.

As used herein, "thermostable" refers to an enzyme which is stable and active at temperatures as great as preferably between about 90-100°C and more preferably between about 70-980C to heat as compared, for example, to a non-thermostable form of an enzyme with a similar activity. For example, a thermostable nucleic acid polymerase derived from thermophilic organisms such as *P. furiosus*, *M. jannaschii*, *A. fulgidus* or *P. horikoshii* are more stable and active at elevated temperatures as compared to a nucleic acid polymerase from *E. coli*. A representative thermostable nucleic acid polymerase isolated from *P. furiosus* (*Pfu*) is described in Lundberg et al., 1991, Gene, 108:1-6. Additional representative temperature stable polymerases include, e.g., polymerases extracted from the thermophilic bacteria *Thermus flavus, Thermus ruber, Thermus thermophilus, Bacillus stearothermophilus* (which has a somewhat lower temperature optimum than the others listed), *Thermus lacteus, Thermus rubens, Thermotoga maritima,* or from thermophilic archaea *Thermococcus litoralis*, and *Methanothermus fervidus*.

Temperature stable polymerases are preferred in a thermocycling process wherein double stranded nucleic acids are denatured by exposure to a high temperature (about 95° C) during the PCR cycle.

As used herein, the term "template DNA molecule" refers to that strand of a nucleic acid from which a complementary nucleic acid strand is synthesized by a DNA polymerase, for example, in a primer extension reaction.

As used herein, the term "template dependent manner" is intended to refer to a process that involves the template dependent extension of a primer molecule (e.g., DNA synthesis by DNA polymerase). The term "template dependent manner" refers to polynucleotide synthesis of RNA or DNA wherein the sequence of the newly synthesized strand of polynucleotide is dictated by the well-known rules of complementary base pairing (see, for example, Watson, J. D. et al., In: Molecular Biology of the Gene, 4th Ed., W. A. Benjamin, Inc., Menlo Park, Calif. (1987)).

As used herein, an "amplified product" refers to the double stranded polynucleotide population at the end of a PCR amplification reaction. The amplified product contains the original polynucleotide template and polynucleotide synthesized by DNA polymerase using the polynucleotide template during the PCR reaction.

As used herein, "polynucleotide template" or "target polynucleotide template" or "template" refers to a polynucleotide containing an amplified region. The "amplified region," as used herein, is a region of a polynucleotide that is to be, for example, synthesized by polymerase chain reaction (PCR). For example, an amplified region of a polynucleotide template resides between two sequences, to which two PCR primers are complementary.

As used herein, the term "primer" refers to a single stranded DNA or RNA molecule that can hybridize to a polynucleotide template and prime enzymatic synthesis of a second polynucleotide strand. A primer useful according to the invention is between 10 to 100 nucleotides in length, preferably 17-50 nucleotides in length and more preferably 17-45 nucleotides in length.

"Complementary" refers to the broad concept of sequence complementarity between regions of two polynucleotide strands or between two nucleotides through base-pairing. It is known that an adenine nucleotide is capable of forming specific hydrogen bonds ("base pairing") with a nucleotide which is thymine or uracil. Similarly, it is known that a cytosine nucleotide is capable of base pairing with a guanine nucleotide.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. In contrast, the

term "modified" or "mutant" refers to a gene or gene product which displays altered characteristics when compared to the wild-type gene or gene product. For example, a mutant DNA polymerase in the present invention is a DNA polymerase which exhibits a reduced uracil detection activity.

As used herein, "reduced base analog detection" refers to a DNA polymerase, with a reduced ability to recognize a base analog, for example, uracil or inosine, present in a DNA template. In this context, mutant DNA polymerase with "reduced" base analog detection activity is a DNA polymerase mutant having a base analog detection activity which is lower than that of the wild-type enzyme. In the case of a mutant DNA polymerase fusion the activity of a mutant DNA polymerase may be compared to the corresponding non-fusion DNA polymerase, i.e., having less than 10% (e.g., 9.9%, 9%, 8%, 6%, 4%, 2% or less than 1%) of the base analog detection activity of that of the wild-type enzyme. Base analog detection activity may be determined according to the assays similar to those described for the detection of DNA polymerases having a reduced uracil detection activity as described in Greagg et al. (1999) Proc. Natl. Acad. Sci. 96, 9045-9050. Alternatively, "reduced" base analog detection refers to a mutant DNA polymerase with a reduced ability to recognize a base analog, the "reduced" recognition of a base analog being evident by an increase in the amount of >10Kb PCR of at least 10%, preferably 50%, more preferably 90%, most preferably 99% or more, as compared to a wild type DNA polymerase without a reduced base analog detection activity. The amount of a > 10Kb PCR product is measured either by spectorophotometer-absorbance assays of gel eluted > 10Kb PCR DNA product or by fluorometric analysis of > 10Kb PCR products in an ethidium bromide stained agarose electrophoresis gel using, for example, a Molecular Dynamics (MD) FluorImager™ (Amersham Biosciences, catalogue #63-0007- 79).

As used herein, "reduced uracil detection" refers to a DNA polymerase with a reduced ability to recognize a uracil base present in a DNA template. In this context, mutant DNA polymerase with "reduced" uracil detection activity is a DNA polymerase mutant having a uracil detection activity which is lower than that of the wild-type enzyme, i.e., having less than 10% (e.g., 9.9%l, 9%, 8%, 6%, 4%, 2% or less than 1%) of the uracil detection activity of that of the wild-type enzyme. Uracil detection activity may be determined according to the

assays described in Greagg et al. (1999) Proc. Natl. Acad. Sci. 96, 9045-9050. Alternatively, "reduced" uracil detection refers to a mutant DNA polymerase with a reduced ability to recognize uracil, the "reduced" recognition of uracil being evident by an increase in the amount of >10Kb PCR of at least 10%, preferably 50%, more preferably 90%, most preferably 99% or more, as compared to a wild type DNA polymerase without a reduced uracil detection activity. The amount of a > 10Kb PCR product is measured either by spectorophotometer-absorbance assays of gel eluted > 10Kb PCR DNA product or by fluorometric analysis of > 10Kb PCR products in an ethidium bromide stained agarose electrophoresis gel using, for example, a Molecular Dynamics (MD) FluorImager™ (Amersham Biosciences, catalogue #63-0007- 79).

As used herein, "chemically modified" refers to a nucleic acid that is chemically or biochemically modified or contains non-natural or derivatized nucleotide bases. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (e.g. methyl phosphonates, phosphorodithioates, etc.), pendent moieties (e.g., polypeptides), intercalators, (e.g. acridine, psoralen, etc.) chelators, alkylators, and modified linkages (e.g. alpha anomeric nucleic acids, etc.) Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

As used herein, a "PCR enhancing factor" or a "Polymerase Enhancing Factor" (PEF) refers to a complex or protein possessing polynucleotide polymerase enhancing activity including, but not limited to, PEF, dUTPase, ssbPCNA, RFC, helicases etc (Hogrefe et al., 1997, Strategies 10:93-96; and U.S. Patent No. 6,183,997, both of which are hereby incorporated by reference). A "PCR enhancing factor" also includes non-protein factors, for example DMSO and betaine.

The invention also contemplates mutant archael DNA polymerases in combination with accessory factors, for example as described in U.S. 6,333,158, and WO 01/09347 A2, hereby incorporated by reference in their entirety.

As used herein, "additive" refers to a PCR enhancing additive, including but not limited to, *Pfu* dUTPase (PEF), PCNA, RPA, ssb, antibodies, DMSO, betaine, or 3'-5' exonuclease (e.g., *Pfu* G387P).

The invention also provides for kits for performing at high pH a method selected from the group consisting of: DNA synthesis; cloning of a DNA synthesis product; sequencing DNA; and linear or exponential PCR amplification comprising a DNA polymerase fusion and packaging materials therefore. The kits of the invention may include a high pH buffer and/or a PCR enhancing factor and/or an additive.

As used herein, a high pH buffer refers to a buffer that has a pH greater than 9. As used herein, "high pH" refers to a pH that is greater than 9. A "high pH" is preferably 10 or more, for example 11, 12, 13 or 14. A "high pH" includes any pH greater than 9 and up to a pH of 14, for example a pH of 9.1, 9.5, 9.8, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, or 14 is a "high pH" according to the invention.

In a preferred embodiment the high pH buffer is a standard PCR reaction buffer, for example cloned *Pfu* reaction buffer described in Example 3, but wherein the buffering component is at a high pH (i.e., 9.1-14). For example, a buffering component of the invention is 30 mM Tris [Tris(hydroxymethyl) aminomethane] at a pH of 10.0 or 11.8. The pH of the buffering component in standard PCR reaction buffers is from 8.3 – 8.8. The buffering component is used at a concentration from 1mM to 1M in the final PCR reaction and is at a pH from 9.1-14. The highly alkaline buffer for PCR reactions is used with the fusion DNA polymerases or fusion DNA polymerase blends of the invention. A buffering component of the present invention includes, but is not limited to, Tris, Tricine, bicine, Bis-Tris, CAPS, EPPS, HEPES, MES, MOPS, PIPES, TAPS and TES.

As used herein "FEN-1 nuclease" refers to thermostable FEN-1 endonucleases useful according to the invention and includes, but is not limited to, FEN-1 endonuclease purified

from the "hyperthermophiles", e.g., from *M. jannaschii*, *P. furiosus* and *P. woesei*. See U.S. Patent No. 5,843,669, hereby incorporated by reference.

According to the methods of the present invention, the addition of FEN-1 in the amplification reaction dramatically increases the efficiency of the multi-site mutagenesis. 400 ng to 4000 ng of FEN-1 may be used in each amplification reaction. Preferably 400-1000 ng, more preferably, 400-600 ng of FEN-1 is used in the amplification reaction. In a preferred embodiment of the invention, 400 ng FEN-1 is used.

As used herein, "Thermus DNA ligase" refers to a thermostable DNA ligase that is used in the multi-site mutagenesis amplification reaction to ligate the mutant fragments synthesized by extending each mutagenic primer so as to form a circular mutant strand. Tth and Taq DNA ligase require NAD as a cofactor.

Preferably, 1-20 U DNA ligase is used in each amplification reaction, more preferably, 2-15 U DNA ligase is used in each amplification reaction.

In a preferred embodiment, 15 U Taq DNA ligase is used in an amplification reaction. Taq DNA ligase cofactor NAD is used at a concentration of 0-1 mM, preferably between 0.02-0.2 mM, more preferably at 0.1 mM.

As used herein, a "blend" refers to a combination of two or more DNA polymerases comprising at least one DNA polymerase fusion and at least one non-fusion DNA polymerase (see Example 2). The invention contemplates a "blend" wherein at least one of said fusion or non-fusion DNA polymerase is thermostable, is an archael or eubacterial DNA polymerase and/or is a Pfu DNA polymerase. The ratio of DNA polymerase enzymes in a "blend" comprising one fusion and one non-fusion polymerase is in the range of 1:1-1:5-5:1, or 1:1-1:10-10:1, or 1:1-1:25-25:1 or 1:1-1:100-100:1 . For embodiments wherein a "blend" comprises one fusion DNA polymerase and two non-fusion polymerases the ratio of the first non-fusion DNA polymerase to the second non-fusion DNA polymerase is in the range of 1:1-1:5-5:1, or 1:1-1:10-10:1, or 1:1-1:25-25:1 or 1:1-1:100-100:1. A "blend" of the invention has a >10% increase in one or more of the following activities (using the assays described hereinbelow) as compared to the non-fusion component of the blend using a

genomic and / or plasmid template.: processivity, efficiency, template length amplification capability, GC-rich target amplification efficiency, specificity, thermostability; intrinsic hot start capability, proofreading activity, fidelity, DNA binding activity, strand displacement activity, nucleotide binding and recognition, and salt resistance. A blend of the invention will also have a >10% decrease as compared to the non-fusion blends using genomic and / or plasmid template in one or more of the following activities (assayed as described hereinbelow): amplification slippage on templates with tri-nucleotide repeat stretches or DNA polymerase activity at room temperature. In one embodiment, a "blend" of the invention has an extension time in a PCR reaction that is decreased by 5 sec, preferably 15 sec and more preferably 45 sec or more, as compared to the extension time observed in the presence of the non-fusion component of the blend alone. In another embodiment, a "blend" of the invention has a decrease in the number of amplification cycles for PCR of 1, 1-5 or 5 or more cycles, as compared to the non-chimeric component of the blend alone. In another embodiment, fewer units (.001, .01, .1 or 1 or more) of a "blend" of the invention are useful in an application of the invention as compared to the non-fusion component of the blend.

A blend may also include a PCR enhancing factor and/or an additive, as described herein.

The invention also relates to compositions made for carrying out the methods of the invention and compositions made while carrying out the methods of the invention. Such compositions may comprise one or more components selected from the group consisting of one or more polymerases of the invention, one or more nucleotides, one or more templates, one or more reaction buffers or buffering salts, one or more primers, one or more nucleic acid products made by the methods of the invention and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: 6kb human βeta globin genomic DNA target amplified with a 15 second per kb extension time (1 minute 30 second total extension time). The PCR reaction buffer consisted of 1X cloned *Pfu* buffer using a 30 mM Tris pH gradient from 5.0 to 10.0. The chimeric

DNA polymerase blend was composed of A; 0.25U chimeric *Pfu* DNA polymerase and 2.5 U *Pfu* Turbo for a total of 2.75 U / reaction and B; 0.25U chimeric *Pfu* DNA polymerase and 5.0 U *Pfu* Turbo for a total of 5.25 U / reaction. M is 1kb DNA marker (Stratagene).

Figure 2: 6kb human βeta globin genomic DNA target amplified with a 15 second per kb extension time (1 minute 30 second total extension time). The PCR reaction buffer consisted of 1X cloned *Pfu* buffer using a 30 mM Tris pH gradient from 9.5 to 12.0. The chimeric DNA polymerase blend was composed of 0.25U chimeric *Pfu* DNA polymerase and 2.5 U *Pfu* Turbo for a total of 2.75 U / reaction. M is 1kb DNA marker (Stratagene).

Figure 3: Comparison of high pH reaction buffers and 1.5X cloned *Pfu* reaction buffer for the 19 kb beta globin genomic target. Lanes 1 and 2 are with the pH 10 buffer. Lanes 3 and 4 are with the pH 11 buffer. Lanes 5 and 6 are with 1.5X cloned *Pfu* reaction buffer. Lanes 1, 3 and 5 were amplified with the chimeric DNA polymerase blend that was composed of 0.25U chimeric *Pfu* DNA polymerase and 2.5 U *Pfu* Turbo for a total of 2.75 U / reaction. Lanes 2, 4, & 6 were amplified with the chimeric DNA polymerase blend that was composed of 0.25U chimeric *Pfu* DNA polymerase and 5.0 U *Pfu* Turbo for a total of 5.25 U / reaction. M is 1kb DNA marker (Stratagene). A 30 second per kb extension time was used.

Figure 4: Comparison of the chimeric *Pfu* DNA polymerase / *Pfu* Turbo DNA polymerase blend and the chimeric *Pfu* DNA polymerase in the high pH PCR reaction buffer and Herculase DNA polymerase in Herculase PCR reaction buffer for the PCR amplification of the 19 kb beta globin genomic target. Lanes 1 to 4 used the pH 10 PCR reaction buffer. Lanes 5 to 8 used Herculase PCR reaction buffer. Lane 1 was amplified with the chimeric DNA polymerase blend that was composed of 0.25U chimeric *Pfu* DNA polymerase and 2.5 U *Pfu* Turbo for a total of 2.75 U / reaction. Lane 2 was amplified with the chimeric DNA polymerase blend that was composed of 0.25U chimeric *Pfu* DNA polymerase and 5.0 U *Pfu* Turbo for a total of 5.25 U / reaction. Lane 3 was amplified with 0.83U of the Pfu chimeric DNA polymerase. Lane 4 was amplified with 1.3U of the chimeric DNA polymerase. Lanes 5 and 6 were amplified with 5.0U of Herculase DNA polymerase with out DMSO. Lanes 7 and 8 were amplified with 5.0U of Herculase DNA polymerase with 3% DMSO. A 30 second per kb extension time was used. M is the Lambda / Hind III DNA marker (Stratagene).

Figure 5: Unit titration of chimeric Pfu DNA polymerase in high pH PCR reaction buffer and performance comparison to Herculase DNA polymerase and KOD hot start for the amplification of the 19kb human beta globin with an extension time of 30 seconds per kb. #1-4, chimeric Pfu-Sso7d DNA polymerase in pH 10.0 PCR reaction buffer. #1- 0.25U; #2- 0.5U; #3- 0.83U; #4- 1.3U. #5-6, 5.0U of Herculase DNA polymerase in 1X Herculase PCR reaction buffer and 3% DMSO. #7-8, KOD hot start DNA polymerase in KOD hot start DNA polymerase PCR reaction buffer. #7- 1.25U; #8- 2.5U. M is the Lambda / Hind III DNA marker (Stratagene).

Figure 6: Performance comparison of chimeric Pfu DNA polymerase in the pH 10.0 PCR reaction buffer and KOD hot start in KOD hot start PCR reaction buffer for the amplification of 900bp Human alpha-1 antitrypsin (Hα1AT) with a 1 second total extension time. #1-2, chimeric Pfu-Sso7d DNA polymerase in pH 10.0 PCR reaction buffer. #3-4, Pfu-Sso7d DNA polymerase in pH 11.8 PCR reaction buffer. #5-6, 1.0U KOD hot start in KOD hot start PCR reaction buffer. #1- 0.5U; #2- 0.83U; #3- 0.5U; #4- 0.83U. M- 1kb DNA marker (Stratagene).

Figure 7: PCR performance comparison of chimeric Pfu-Sso7d DNA polymerase in pH 10.0 PCR reaction buffer and KOD hot start DNA polymerase in KOD hot start PCR reaction buffer for the amplification of 2.6kb Human alpha-1 antitrypsin (Hα1AT) with an extension time of 2 seconds per kb (5 second total extension time). #1-3, Pfu-Sso7d DNA polymerase in pH 10.0 PCR reaction buffer. #4-5, KOD hot start DNA polymerase in KOD hot start PCR reaction buffer. #1- 0.5U; #2- 0.83U; #3- 1.3U; #4- 1.25U; #5- 2.5U. M-1kb DNA ladder (Stratagene).

Figure 8: PCR performance comparison of chimeric Pfu-Sso7d DNA polymerase in pH 10.0 PCR reaction buffer and KOD hot start in KOD hot start PCR reaction buffer for the amplification of 6kb human beta globin with an extension time of 10 seconds per kb. #1-3, chimeric Pfu-Sso7d DNA polymerase in pH 10.0 PCR reaction buffer. #4-5. KOD hot start DNA polymerase in KOD hot start PCR reaction buffer. #1- 0.5U; #2- 0.83U; #3- 1.3U; #4- 1.25U; #5- 2.5U. M- 1kb DNA ladder (Stratagene).

Figure 9: PCR performance comparison of chimeric Pfu-Sso7d DNA polymerase in pH 10.0 PCR reaction buffer and KOD hot start in KOD hot start PCR reaction buffer for the amplification of 2.6kb Hα1AT with an extension of 30 seconds per kb (1 minute 18 seconds total extension time). #1-3, Pfu-Sso7d DNA polymerase. #4-5, KOD hot start DNA polymerase. #1- 0.5U; #2- 0.83U; #3- 1.3U; #4- 1.25U; #5- 2.5U. M- 1kb DNA ladder (Stratagene).

Figure 10: Oligonucleotide Primers for QuickChange Mutagenesis (SEQ ID Nos: 6-14)

Figure 11: (a) dUTP incorporation of V93E and V93R mutants compared to wild type *Pfu* DNA polymerase.

(b) PCR Amplification of *Pfu* V93R mutant extract in the presence of 100% dUTP.

Figure 12: Comparison of the efficacy of "long" PCR amplification of *Pfu* DNA polymerase mutants and wt enzyme.

Figure 13: 13A. DNA sequence of mutant archeael DNA polymerases

13B. Amino acid sequence of mutant archeael DNA polymerases

Figure 14: DNA and Amino acid sequence of mutant Tgo DNA polymerase DNA

Figure 15: dUTP incorporation of *Pfu* mutants compared to wild type *Pfu* DNA polymerase

15A. dUTP incorporation of *Pfu* mutants V93W, V93Y, V93M, V93K and V93R compared to wild type *Pfu* DNA polymerase

15B. dUTP incorporation of the *Pfu* V93D and V93R mutants compared to wild type *Pfu* DNA polymerase.

15C. dUTP incorporation of the *Pfu* V93N and V93G mutant compared to wild type *Pfu* DNA polymerase

Figure 16: DNA polymerase activity of N-terminal Pfu DNA polymerase truncation mutants.

Figure 17: shows the sequence of

A. HMf-like protein

B. HMf-like protein-Taq fusion

C. HMf-like protein-Taq fusion

D. Pfu WT-HMf like protein fusion

E. Pfu WT-HMf like protein fusion

F. Pfu-V93 R or E-HMf-like protein fusion

G. Pfu-V93 R or E-HMf-like protein fusion

H. Pfu-G387P/V93 R or E-HMf-like protein fusion

I. Pfu-G387P/V93 R or E-HMf-like protein fusion

J. Pfu-D141A/E143A/V93 R or E-HMf-like protein fusion

K. Pfu-D141A/E143A/V93 R or E-HMf-like protein fusion

L. KOD-HMf-like protein fusion

M. KOD-HMf-like protein fusion

N. HMf-like protein- Vent fusion

O. HMf-like protein- Vent fusion

P. HMf-like protein- DeepVent fusion

Q. HMf-like protein- DeepVent fusion

R. HMf-like protein- JDF3 fusion

S. HMf-like protein- JDF3 fusion

T. PCNA

U. PCNA-Taq fusion

V. PCNA-Taq fusion

W.PCNA-PfuWT fusion

X. PCNA-PfuWT fusion

Y. Pfu-V93 R or E-PCNA fusion

Z. Pfu-V93 R or E-PCNA fusion

AA. Pfu-G387P/V93 R or E-PCNA fusion

BB. Pfu-G387P/V93 R or E-PCNA fusion

CC. Pfu-D141A/E143A/V93 R or E-PCNA fusion

DD. Pfu-D141A/E143A/V93 R or E-PCNA fusion

EE. KOD-PCNA fusion

FF. KOD-PCNA protein fusion

GG. PCNA- Vent fusion

HH. PCNA- Vent fusion

II. PCNA- DeepVent fusion

JJ. PCNA- DeepVent fusion

KK. PCNA- JDF3 fusion

LL. PCNA- JDF3 fusion

MM. Sac7d

NN. Sac7d -Taq fusion

OO. Sac7d -Taq fusion

PP . Sac7d -PfuWT fusion

QQ. Sac7d -PfuWT fusion

RR. Pfu-V93 R or E- Sac7d -like protein fusion

SS. Pfu-V93 R or E- Sac7d fusion

TT. Pfu-G387P/V93 R or E- Sac7d fusion

UU. Pfu-G387P/V93 R or E- Sac7d fusion

VV. Pfu-D141A/E143A/V93 R or E-Sac7d fusion

WW. KOD- Sac7d fusion

XX. KOD- Sac7d protein fusion

YY. Sac7d - Vent fusion

ZZ. Sac7d - Vent fusion

AAA. Sac7d - DeepVent fusion

BBB. Sac7d - DeepVent fusion

CCC. Sac7d- JDF3 fusion

DDD. Sac7d- JDF3 fusion

EEE. Sso7D

FFF. Sso7D -Taq fusion

GGG.Sso7D -PfuWT fusion

HHH. Pfu-G387P/V93 R or E- Sso7D fusion

III. Pfu-G387P/V93 R or E- Sso7D fusion

JJJ. Pfu-D141A/E143A/V93 R or E- Sso7D fusion

KKK. KOD- Sso7D fusion

LLL. KOD- Sso7D fusion

MMM. Sso7D - Vent fusion

NNN. Sso7D - Vent fusion

OOO. Sso7D - DeepVent fusion

PPP. Sso7D - DeepVent fusion

QQQ. Sso7D - JDF3 fusion

RRR. Sso7D - JDF3 fusion

Figure 18: HhH motif Sequences

(a) Motifs conserved between topo V, RecA, and leucine-responsive regulator signature sequences. Topo V amino acid region 236-298 made no hits in databases and is not shown. A short region between positions 677-695 connecting repeats G and H and the 19-aa residues at the end of the sequence is not shown for simplicity. Invariant residues are shown on blue backgrounds with white lettering. Conservative positions are highlighted on the yellow background. (b) Structure of topo V HhH motifs. Backgrounds of Lys-68 and Lys-72 of -pol and corresponding positions in C and G repeats of topo V are colored cyan and magenta, respectively. Secondary structures in a and b were predicted by using JPRED. Cylinders represent -helices, and lines between them (b) represent –hairpins.. MkTpV, M. kandleri topo V; HTH asnC, the three-element fingerprint that provides a signature for the HTH motif of the asnC bacterial regulatory proteins; HTH SS, secondary structure of the HTH motif; A-L, topo V's HhH repeats; EcRuvA, E. coli RuvA protein, HsPolB, human polymerase ; TaqPol, T. aquaticus polymerase I; HhH SS, secondary structure of HhH motifs. ALSCRIPT (Pargellis et al. (1988) J. Biol. Chem. 263, 7678-7685) was used to illustrate the alignments. Cited from Belova et al., 2001, Proc. Natl. Acad. Sci. USA, 98:6015).

Figure 19: Additional sequences of the invention

Figure 20: DNA and Amino acid sequence of wild type Pfu DNA polymerase

DETAILED DESCRIPTION

The present invention discloses DNA polymerase fusions for use in PCR, DNA sequencing and mutagenesis protocols at high pH. The invention allows for PCR reactions with shorter extension times that will facilitate PCR amplification of genomic DNA templates and improve the efficacy of long PCR.

I. DNA Polymerases according to the Invention

The invention provides for a DNA polymerase fusion. The DNA polymerase fusions, useful according to the invention, can be with or without 3'-5' exonuclease activity, i.e., proofreading or non-proofreading, and are preferably thermostable. The invention provides for DNA polymerase fusions that harbor one or more mutations that modify one or more activities normally found in the wild-type DNA polymerase that is not a fusion, as defined herein.

Additional nucleic acid polymerases useful according to the invention are listed below.

A. Bacteriophage DNA polymerases (Useful for 37°C assays):

Bacteriophage DNA polymerases are devoid of 5' to 3' exonuclease activity, as this activity is encoded by a separate polypeptide. Examples of suitable DNA polymerases are T4, T7, and ϕ29 DNA polymerase. The enzymes available commercially are: T4 (available from many sources e.g., Epicentre) and T7 (available from many sources, e.g. Epicentre for unmodified and USB for 3' to 5' exo⁻ T7 "Sequenase" DNA polymerase).

B. Archaeal DNA polymerases:

There are 2 different classes of DNA polymerases which have been identified in archaea: 1. Family B/pol I type (homologs of *Pfu* from *Pyrococcus furiosus*) and 2. pol II type (homologs of *P. furiosus* DP1/DP2 2-subunit polymerase). DNA polymerases from both classes have been shown to naturally lack an associated 5' to 3' exonuclease activity and to possess 3' to 5' exonuclease (proofreading) activity. Suitable DNA polymerases (pol I or pol II) can be derived from archaea with optimal growth temperatures that are similar to the desired assay temperatures.

Thermostable archaeal DNA polymerases are isolated from Pyrococcus species (furiosus, species GB-D, woesii, abysii, horikoshii), Thermococcus species (kodakaraensis KOD1, litoralis, species 9 degrees North-7, species JDF-3, gorgonarius), Pyrodictium occultum, and Archaeoglobus fulgidus. It is estimated that suitable archaea would exhibit

maximal growth temperatures of >80-85^{0}C or optimal growth temperatures of >70-80^{0}C. Appropriate PCR enzymes from the archaeal pol I DNA polymerase group are commercially available, including *Pfu* (Stratagene), KOD (Toyobo), Pfx (Life Technologies, Inc.), Vent (New England BioLabs), Deep Vent (New England BioLabs), Tgo (Roche), and Pwo (Roche).

Additional archaea DNA polymerases related to those listed above are described in the following references: Archaea: A Laboratory Manual (Robb, F.T. and Place, A.R., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1995 and *Thermophilic Bacteria* (Kristjansson, J.K.,ed.) CRC Press, Inc., Boca Raton, Florida, 1992.

The invention therefore provides for thermostable archaeal DNA polymerases of either Family B/pol I type or pol II type as well as mutants or derivatives thereof.

Table 1. ACCESSION INFORMATION FOR CLONED FAMILY B POLYMERASES

Vent Thermococcus litoralis

ACCESSION AAA72101

PID g348689

VERSION AAA72101.1 GI:348689

DBSOURCE locus THCVDPE accession M74198.1

THEST THERMOCOCCUS SP. (STRAIN TY)

ACCESSION O33845

PID g3913524

VERSION O33845 GI:3913524

DBSOURCE swissprot: locus DPOL_THEST, accession O33845

Pab Pyrococcus abyssi

ACCESSION P77916

PID g3913529

VERSION P77916 GI:3913529

DBSOURCE swissprot: locus DPOL_PYRAB, accession P77916

PYRHO Pyrococcus horikoshii

ACCESSION O59610

PID g3913526

VERSION O59610 GI:3913526

DBSOURCE swissprot: locus DPOL_PYRHO, accession O59610

PYRSE PYROCOCCUS SP. (STRAIN GE23)

ACCESSION P77932

PID g3913530

VERSION P77932 GI:3913530

DBSOURCE swissprot: locus DPOL_PYRSE, accession P77932

DeepVent Pyrococcus sp.

ACCESSION AAA67131

PID g436495

VERSION AAA67131.1 GI:436495

DBSOURCE locus PSU00707 accession U00707.1

Pfu Pyrococcus furiosus

ACCESSION P80061

PID g399403

VERSION P80061 GI:399403

DBSOURCE swissprot: locus DPOL_PYRFU, accession P80061

JDF-3 Thermococcus sp.

Unpublished

Baross gi|2097756|pat|US|5602011|12 Sequence 12 from patent US 5602011

9degN THERMOCOCCUS SP. (STRAIN 9ON-7).

ACCESSION Q56366

PID g3913540

VERSION Q56366 GI:3913540

DBSOURCE swissprot: locus DPOL_THES9, accession Q56366

KOD Pyrococcus sp.

ACCESSION BAA06142

PID g1620911

VERSION BAA06142.1 GI:1620911

DBSOURCE locus PYWKODPOL accession D29671.1

Tgo Thermococcus gorgonarius.

ACCESSION 4699806

PID g4699806

VERSION GI:4699806

DBSOURCE pdb: chain 65, release Feb 23, 1999

THEFM Thermococcus fumicolans

ACCESSION P74918

PID g3913528

VERSION P74918 GI:3913528

DBSOURCE swissprot: locus DPOL_THEFM, accession P74918

METTH Methanobacterium thermoautotrophicum

ACCESSION O27276

PID g3913522

VERSION O27276 GI:3913522

DBSOURCE swissprot: locus DPOL_METTH, accession O27276

Metja Methanococcus jannaschii

ACCESSION Q58295

PID g3915679

VERSION Q58295 GI:3915679

DBSOURCE swissprot: locus DPOL_METJA, accession Q58295

POC Pyrodictium occultum

ACCESSION B56277

PID g1363344

VERSION B56277 GI:1363344

DBSOURCE pir: locus B56277

ApeI Aeropyrum pernix

ACCESSION BAA81109

PID g5105797

VERSION BAA81109.1 GI:5105797

DBSOURCE locus AP000063 accession AP000063.1

ARCFU Archaeoglobus fulgidus

ACCESSION O29753

PID g3122019

VERSION O29753 GI:3122019

DBSOURCE swissprot: locus DPOL_ARCFU, accession O29753

Desulfurococcus sp. Tok.

ACCESSION 6435708

PID g64357089

VERSION GT:6435708

DBSOURCE pdb. chain 65, release Jun 2, 1999

C. Eubacterial DNA polymerases:

There are 3 classes of eubacterial DNA polymerases, pol I, II, and III. Enzymes in the Pol I DNA polymerase family possess 5' to 3' exonuclease activity, and certain members also exhibit 3' to 5' exonuclease activity. Pol II DNA polymerases naturally lack 5' to 3' exonuclease activity, but do exhibit 3' to 5' exonuclease activity. Pol III DNA polymerases represent the major replicative DNA polymerase of the cell and are composed of multiple

subunits. The pol III catalytic subunit lacks 5' to 3' exonuclease activity, but in some cases 3' to 5' exonuclease activity is located in the same polypeptide.

There are no commercial sources of eubacterial pol II and pol III DNA polymerases.

There are a variety of commercially available Pol I DNA polymerases, some of which have been modified to reduce or abolish 5' to 3' exonuclease activity.

Suitable thermostable pol I DNA polymerases can be isolated from a variety of thermophilic eubacteria, including *Thermus species* and *Thermotoga maritima* such as *Thermus aquaticus (Taq), Thermus thermophilus (Tth)* and *Thermotoga maritima* (Tma UlTma).

Additional eubacteria related to those listed above are described in *Thermophilic Bacteria* (Kristjansson, J.K.,ed.) CRC Press, Inc., Boca Raton, Florida, 1992.

The invention further provides for chimeric or non-chimeric DNA polymerases that are chemically modified according to methods disclosed in U.S. Patent No. 5,677,152, 6,479,264 and 6,183, 998, the contents of which are hereby incorporated by reference in their entirety.

II. PREPARING MUTANT DNA POLYMERASES

According to the invention, DNA polymerases can be generated from any DNA polymerase either wild-type or modified to contain one or more mutations, including but not limited to, one or more point mutations, N- and/or C- truncations, internal deletion or insertion that would cause the DNA polymerase to behave differently than the wild-type polymerase. DNA polymerase mutations useful to the invention include, but are not limited to, mutations that confer base analog or uracil insensitivity, increase fidelity, eliminate 3'-5' exonuclease activity or eliminate 5'-3' exonuclease activity or reduce polymerase activity. Specific examples of useful mutations or truncations include but are not limited to, V93R,K,E,D in *Pfu* DNA polymerase, which confer uracil insensitivity, D141A / E143A in *Pfu* DNA polymerase, which eliminates 3'-5' exonuclease activity, and the N-terminal truncation of *Taq* DNA polymerase to eliminate 5'-3' exonuclease activity(Klen*Taq*).

Methods for generating DNA polymerase mutants are described below and other methods are known in the art.

GENETIC MODIFICATIONS - MUTAGENESIS

Direct comparison of DNA polymerases from diverse organisms indicates that the domain structure of these enzymes is highly conserved and in many instances, it is possible to assign a particular function to a well-defined domain of the enzyme. For example, the six most conserved C-terminal regions, spanning approximately 340 amino acids, are located in the same linear arrangement and contain highly conserved motifs that form the metal and dNTP binding sites and the cleft for holding the DNA template and are therefore essential for the polymerization function. In another example, the three amino acid regions containing the critical residues in the E. coli DNA polymerase I involved in metal binding, single-stranded DNA binding, and catalysis of the 3'-5' exonuclease reaction are located in the amino-terminal half and in the same linear arrangement in several prokaryotic and eukaryotic DNA polymerases. The location of these conserved regions provides a useful model to direct genetic modifications for preparing mutant DNA polymerase with modified activities whilst conserving essential functions e.g. DNA polymerization and proofreading activity.

For example, a mutant DNA polymerase can be generated by genetic modification (e.g., by modifying the DNA sequence of a wild-type DNA polymerase). A number of methods are known in the art that permit the random as well as targeted mutation of DNA sequences (see for example, Ausubel et. al. Short Protocols in Molecular Biology (1995) 3rd Ed. John Wiley & Sons, Inc.). In addition, there are a number of commercially available kits for site-directed mutagenesis, including both conventional and PCR-based methods. Examples include the EXSITE™ PCR-Based Site-directed Mutagenesis Kit available from Stratagene (Catalog No. 200502) and the QUIKCHANGE™ Site-directed mutagenesis Kit from Stratagene (Catalog No. 200518), and the CHAMELEON® double-stranded Site-directed mutagenesis kit, also from Stratagene (Catalog No. 200509).

In addition mutant DNA polymerases may be generated by insertional mutation or truncation (N-terminal, internal or C-terminal) according to methodology known to a person skilled in the art.

Older methods of site-directed mutagenesis known in the art rely on sub-cloning of the sequence to be mutated into a vector, such as an M13 bacteriophage vector, that allows the isolation of single-stranded DNA template. In these methods, one anneals a mutagenic primer (i.e., a primer capable of annealing to the site to be mutated but bearing one or more mismatched nucleotides at the site to be mutated) to the single-stranded template and then polymerizes the complement of the template starting from the 3' end of the mutagenic primer. The resulting duplexes are then transformed into host bacteria and plaques are screened for the desired mutation.

More recently, site-directed mutagenesis has employed PCR methodologies, which have the advantage of not requiring a single-stranded template. In addition, methods have been developed that do not require sub-cloning. Several issues must be considered when PCR-based site-directed mutagenesis is performed. First, in these methods it is desirable to reduce the number of PCR cycles to prevent expansion of undesired mutations introduced by the polymerase. Second, a selection must be employed in order to reduce the number of non-mutated parental molecules persisting in the reaction. Third, an extended-length PCR method is preferred in order to allow the use of a single PCR primer set. And fourth, because of the non-template-dependent terminal extension activity of some thermostable polymerases it is often necessary to incorporate an end-polishing step into the procedure prior to blunt-end ligation of the PCR-generated mutant product.

The protocol described below accommodates these considerations through the following steps. First, the template concentration used is approximately 1000-fold higher than that used in conventional PCR reactions, allowing a reduction in the number of cycles from 25-30 down to 5-10 without dramatically reducing product yield. Second, the restriction endonuclease Dpn I (recognition target sequence: 5-Gm6ATC-3, where the A residue is methylated) is used to select against parental DNA, since most common strains of E. coli Dam methylate their DNA at the sequence 5-GATC-3. Third, Taq Extender is used in the PCR mix in order to increase the proportion of long (i.e., full plasmid length) PCR products. Finally, *Pfu* DNA polymerase is used to polish the ends of the PCR product prior to intramolecular ligation using T4 DNA ligase.

A non-limiting example for the isolation of non-chimeric mutant DNA polymerases is described in detail as follows:

Plasmid template DNA (approximately 0.5 pmole) is added to a PCR cocktail containing: 1x mutagenesis buffer (20 mM Tris HCl, pH 7.5; 8 mM $MgCl_2$; 40 μg/ml BSA); 12-20 pmole of each primer (one of skill in the art may design a mutagenic primer as necessary, giving consideration to those factors such as base composition, primer length and intended buffer salt concentrations that affect the annealing characteristics of oligonucleotide primers; one primer must contain the desired mutation, and one (the same or the other) must contain a 5' phosphate to facilitate later ligation), 250 μM each dNTP, 2.5 U Taq DNA polymerase, and 2.5 U of Taq Extender (Available from Stratagene; See Nielson et al. (1994) Strategies 7: 27, and U.S. Patent No. 5,556,772). Primers can be prepared using the triester method of Matteucci et al., 1981, J. Am. Chem. Soc. 103:3185-3191, incorporated herein by reference. Alternatively automated synthesis may be preferred, for example, on a Biosearch 8700 DNA Synthesizer using cyanoethyl phosphoramidite chemistry.

The PCR cycling is performed as follows: 1 cycle of 4 min at 94°C, 2 min at 50°C and 2 min at 72°C; followed by 5-10 cycles of 1 min at 94°C, 2 min at 54°C and 1 min at 72°C. The parental template DNA and the linear, PCR-generated DNA incorporating the mutagenic primer are treated with DpnI (10 U) and *Pfu* DNA polymerase (2.5U). This results in the DpnI digestion of the in vivo methylated parental template and hybrid DNA and the removal, by *Pfu* DNA polymerase, of the non-template-directed Taq DNA polymerase-extended base(s) on the linear PCR product. The reaction is incubated at 37°C for 30 min and then transferred to 72°C for an additional 30 min. Mutagenesis buffer (115 ul of 1x) containing 0.5 mM ATP is added to the DpnI-digested, *Pfu* DNA polymerase-polished PCR products. The solution is mixed and 10 ul are removed to a new microfuge tube and T4 DNA ligase (2-4 U) is added. The ligation is incubated for greater than 60 min at 37°C. Finally, the treated solution is transformed into competent E. coli according to standard methods.

Methods of random mutagenesis, which will result in a panel of mutants bearing one or more randomly situated mutations, exist in the art. Such a panel of mutants may then be screened for improved activity such as those exhibiting properties including but not limited to

reduced DNA polymerization activity, 3'-5' exonuclease deficiency, and/or reduced uracil detection activity relative to the wild-type polymerase (e.g., by measuring the incorporation of 10nmoles of dNTPs into polymeric form in 30 minutes in the presence of 200μM dUTP and at the optimal temperature for a given DNA polymerase). An example of a method for random mutagenesis is the so-called "error-prone PCR method". As the name implies, the method amplifies a given sequence under conditions in which the DNA polymerase does not support high fidelity incorporation. The conditions encouraging error-prone incorporation for different DNA polymerases vary, however one skilled in the art may determine such conditions for a given enzyme. A key variable for many DNA polymerases in the fidelity of amplification is, for example, the type and concentration of divalent metal ion in the buffer. The use of manganese ion and/or variation of the magnesium or manganese ion concentration may therefore be applied to influence the error rate of the polymerase.

Genes for desired mutant DNA polymerases generated by mutagenesis may be sequenced to identify the sites and number of mutations. For those mutants comprising more than one mutation, the effect of a given mutation may be evaluated by introduction of the identified mutation to the wild-type gene by site-directed mutagenesis in isolation from the other mutations borne by the particular mutant. Screening assays of the single mutant thus produced will then allow the determination of the effect of that mutation alone.

In one embodiment, the invention provides for blends of two or more DNA polymerases comprising one or more DNA polymerase fusions of the invention with or without an additive as described herein.

In a preferred embodiment, the invention provides for blends of two or more DNA polymerases comprising one or more DNA polymerase fusions and one or more mutant DNA polymerases, at least one of which is derived from *Pfu* DNA polymerase.

In another preferred embodiment, the invention provides for blends of two or more DNA polymerases comprising one or more DNA polymerase fusions and one or more non-chimeric DNA polymerases, at least one of which is derived from *Taq*DNA polymerase.

In another preferred embodiment, the invention provides for a high pH buffer used in PCR amplification reactions with a fusion DNA polymerase or with a blend of a fusion DNA polymerase and a wild type, mutant, or chemically modified DNA polymerase and / or a wild type, mutant, or chemically modified DNA polymerase formulation (see Example 2). As used herein, a "DNA polymerase" formulation is a blend of two or more DNA polymerases, for example, 2,3,4,5 or more, with or without an additive as defined herein.

A person of average skill in the art having the benefit of this disclosure will recognize that DNA polymerases derived from other exo$^+$ DNA polymerases including Vent DNA polymerase, JDF-3 DNA polymerase, Tgo DNA polymerase, KOD DNA polymerase and the like may be suitably used in the subject compositions.

The amino acid and DNA coding sequence of a wild-type *Pfu* DNA polymerase are shown in Figure 20 (Genbank Accession # P80061). A detailed description of the structure and function of *Pfu* DNA polymerase can be found, among other places in U.S. Patent Nos. 5,948,663; 5,866,395; 5,545,552; 5,556,772, all of which are hereby incorporated in their entirety by reference.

The enzyme of the subject composition may comprise DNA polymerases that have not yet been isolated.

The invention provides for blends of two or more DNA polymerases comprising one or more DNA polymerase fusion and one or more mutant or wild type DNA polymerase that is not a fusion.

The invention provides for blends of two or more DNA polymerases comprising one or more DNA polymerase fusions and one or more non-fusion mutant *Pfu* DNA polymerases containing one or more mutations that reduce base analog detection activity as disclosed in the pending U.S. patent application Serial No.: 10/280,962 (Sorge et al.; filed: October 25, 2002) and the pending U.S. patent application Serial No.: 10/298,680 (Sorge et al.; filed November 18, 2002), the contents of which are hereby incorporated in their entirety.

In a preferred embodiment, the blend of two or more DNA polymerases comprises one or more DNA polymerase fusion and one or more non-fusion mutant *Pfu* DNA polymerase of

the invention containing a Valine to Arginine, Valine to Glutamic acid, Valine to Lysine, Valine to Aspartic Acid or Valine to Asparagine substitution at amino acid position 93.

The invention further provides for a blend of two or more DNA polymerases comprising one or more DNA polymerase fusions and one or more non-fusion mutant archaeal DNA polymerases with reduced base analog detection activity that contain a Valine to Arginine, Valine to Glutamic acid, Valine to Lysine, Valine to Aspartic Acid or Valine to Asparagine substitution at amino acid position 93.

A Pfu DNA polymerase mutant with Reduced Uracil Detection can be prepared as follows. Mutations are introduced into *Pfu* DNA polymerase that are likely to reduce uracil detection, while having minimal effects on polymerase or proofreading activity. The DNA template used for mutagenesis contains the *Pfu* pol gene, cloned into pBluescript (pF72 clone described in US 5,489,523). Point mutations are introduced using the QuikChange or the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). With the QuikChange kit, point mutations are introduced using a pair of mutagenic primers (V93E, H, K, R, and N). With the QuikChange Multi kit, specific point mutations are introduced by incorporating one phosphorylated mutagenic primer or by selecting random mutants from a library of *Pfu* V93 variants, created by incorporating a degenerate codon (V93G and L). Clones are sequenced to identify the incorporated mutations.

Valine 93 in *Pfu* DNA polymerase was substituted with Glycine (G), asparagine (N), arginine [R], glutamic acid (E), histidine (H), and leucine (L) using the QuikChange primer sequences listed in Figure 10.

Assessment of the activity of a mutant chimeric or non-chimeric Pfu DNA polymerase is determined as follows.

Partially-purified fusion or non-fusion *Pfu* DNA polymerase preparations exhibiting reduced uracil detection activity (heat-treated bacterial extracts) are assayed for dUTP incorporation during PCR as described in copending application U.S. Serial No. 10/280,962, (Sorge et al., filed October 25, 2002) hereby incorporated by reference in its entirety. In this example, a 2.3kb fragment containing the *Pfu* pol gene was from plasmid DNA using PCR

primers: (F*Pfu*LIC) 5'-gACgACgACAAgATgATTTTAgATgTggAT-3' and (R*Pfu*LIC) 5'-ggAACAAgACCCgTCTAggATTTTTTAATg-3'. Amplification reactions consisted of 1x cloned *Pfu* PCR buffer, 7 ng plasmid DNA, 100ng of each primer, 2.5U of *Pfu* mutant (or wild type *Pfu*), and 200μM each dGTP, dCTP, and dATP. To assess relative dUTP incorporation, various amounts of dUTP (0-400μM) and/or TTP (0-200μM) are added to the PCR reaction cocktail. The amplification reactions were cycled as follows.

Target size (kb)	Target gene	Cycling Parameters
0.9	Hα1AT	(1 cycle) 95°C 2 min (30 cycles) 95°C 5 sec, 58°C 5 sec, 72°C 5 sec (1 cycle) 72°C 7 min
2.6	Hα1AT	(1 cycle) 95°C 2 min (30 cycles) 95°C 20 sec, 58°C 20 sec, 72°C 39 sec. (1 cycle) 72°C 7 min
4	β globin	(1 cycle) 95°C 2 min (30 cycles)95°C 30 sec, 54°C 30 sec, 72°C 1 min (1 cycle) 72°C 7 min
9	β globin	(1 cycle) 95°C 2 min (30 cycles) 95°C 40 sec, 58°C 30 sec, 72°C 4.5 min (1 cycle) 72°C 10 min

12	β globin	(1 cycle) 95°C 2 min (30 cycles) 95°C 40 sec, 58°C 30 sec, 72°C 6 min (1 cycle) 72°C 10 min
17	β globin	(one cycle) 92°C 2 min (10 cycles) 92°C 10 sec, 63°C 30 sec, 68°C 8.5 min (20 cycles) 92°C 10 sec, 63°C 30 sec, 68°C 8.5 min (plus 10 sec/cycle) (one cycle) 68°C 10 min

The invention further provides for a blend of two or more DNA polymerases comprising one or more DNA polymerase fusion and one or more non-fusion mutant archaeal DNA polymerases with a G387P mutant archaeal DNA polymerase with reduced DNA polymerization activity.

The invention further provides for a blend of two or more DNA polymerases comprising one or more DNA polymerase fusions and one or more non-fusion V93 mutant *Pfu* DNA polymerases with reduced uracil detection activity that contain one or more additional mutations that modulates one or more additional activities of V93 *Pfu* DNA polymerases, e.g., DNA polymerization activity or 3'-5' exonuclease activity. In one embodiment, the non-fusion V93 mutant *Pfu* DNA polymerase according to the invention contains one or more mutations that renders the DNA polymerase 3'-5' exonuclease deficient. In another embodiment, the non-fusion V93 mutant *Pfu* DNA polymerase according to the invention contains one or more mutations that reduce the DNA polymerization activity of the non-fusion V93 *Pfu* DNA polymerase.

The invention further provides for a blend of two or more DNA polymerases comprising one or more DNA polymerase fusions and one or more non-fusion V93 mutant *Pfu* DNA polymerases with reduced uracil detection activity that contain one or mutations that reduce DNA polymerization as disclosed in the pending U.S. patent application Serial

No.: 10/035,091 (Hogrefe, et al.; filed: December 21, 2001); the pending U.S. patent application Serial No.: 10/079,241 (Hogrefe, et al.; filed February 20, 2002); the pending U.S. patent application Serial No.: 10/208,508 (Hogrefe et al.; filed July 30, 2002); and the pending U.S. patent application Serial No.: 10/227,110 (Hogrefe et al.; filed August 23, 2002), the contents of which are hereby incorporated in their entirety.

In one embodiment, the invention provides for a V93R/ G387P, V93E/ G387P, V93D/G387P, V93K/G387P or V93N/G387P double mutant *Pfu* DNA polymerase with reduced DNA polymerization activity and reduced uracil detection activity.

The invention further provides for V93R, V93E, V93D, V93K or V93N mutant *Pfu* DNA polymerases with reduced uracil detection activity containing one or more mutations that reduce or eliminate 3'-5' exonuclease activity as disclosed in the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000).

In one embodiment, the invention provides for a non-fusion V93R/D141A/E143A triple mutant *Pfu* DNA polymerase with reduced 3'-5' exonuclease activity and reduced uracil detection activity.

The invention further provides for one or more Pfu DNA polymerases of the invention comprising any combination of one or more mutations that may increase or eliminate base analog detection activity of an archaeal DNA polymerase.

DNA polymerases containing additional mutations are generated by site directed mutagenesis using the DNA polymerases of the invention as a template DNA molecule, for example, the *Pfu* DNA polymerase or Pfu V93R cDNA, according to methods that are well known in the art and are described herein.

The invention contemplates DNA polymerase fusions wherein the DNA polymerase domain of the fusion comprises any of the mutations described herein and known in the art.

Methods used to generate *Pfu* DNA polymerases with reduced DNA polymerization activity of the invention are disclosed in the pending U.S. patent application Serial No.: 10/035,091 (Hogrefe, et al.; filed: December 21, 2001); the pending U.S. patent application

Serial No.: 10/079,241 (Hogrefe, et al.; filed February 20, 2002); the pending U.S. patent application Serial No.: 10/208,508 (Hogrefe et al.; filed July 30, 2002); and the pending U.S. patent application Serial No.: 10/227,110 (Hogrefe et al.; filed August 23, 2002); and the pending U.S. patent application Serial No.: 10/324,846 (Borns et al.; filed December 20, 2002), the contents of which are hereby incorporated in their entirety.

Methods used to generate 3'-5' exonuclease deficient JDF-3 DNA polymerases including the D141A and E143A mutations are disclosed in the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000). A person skilled in the art in possession of the teachings of the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000) would have no difficulty introducing both the corresponding D141A and E143A mutations or other 3'-5' exonuclease mutations into a DNA polymerase of the invention including for example, the non-chimeric V93 *Pfu* DNA polymerase cDNA, as disclosed in the pending U.S. patent application Serial No.: 09/698,341, using established site-directed mutagenesis methodology.

Three 3' to 5' exonuclease motifs have been identified, and mutations in these regions have also been shown to abolish 3' to 5' exonuclease activity in Klenow, ϕ29, T4, T7, and Vent DNA polymerases, yeast Pol α, Pol β, and Pol γ, and Bacillus subtilis Pol III (reviewed in Derbeyshire et al., 1995, Methods. Enzymol. 262:363). Methods for preparing additional DNA polymerase mutants, with reduced or abolished 3' to 5' exonuclease activity, are well known in the art.

Commercially-available enzymes that lack both 5' to 3' and 3' to 5' exonuclease activities include Sequenase (exo$^-$ T7; USB), *Pfu* exo$^-$ (Stratagene), exo$^-$ Vent (New England BioLabs), exo$^-$ DeepVent (New England BioLabs), exo$^-$ Klenow fragment (Stratagene), *Bst* (Bio-Rad), Isotherm (Epicentre), Ladderman (Panvera), KlenTaq1 (Ab Peptides), Stoffel fragment (Perkin-Elmer), ThermoSequenase (USB), and TaqFS (Hoffman-LaRoche), any one of which may be used as the non chimeric DNA polymerase component in the blend of the invention disclosed herein.

In accordance with the invention, in addition to the mutations described above, one or more additional mutations or modifications (or combinations thereof) may be made to the

polymerases of interest. Mutations or modifications of particular interest include those modifications of mutations which (1) eliminate or reduce 5' to 3' exonuclease activity; and (2) reduce discrimination of dideoxynucleotides (that is, increase incorporation of dideoxynucleotides). The 5'-3' exonuclease activity of the polymerases can be reduced or eliminated by mutating the polymerase gene or by deleting the 5' to 3' exonuclease domain. Such mutations include point mutations, frame shift mutations, deletions, and insertions. Preferably, the region of the gene encoding an DNA polymerase activity is deleted using techniques well known in the art. For example, any one of six conserved amino acids that are associated with the 5'-3' exonuclease activity can be mutated. Examples of these conserved amino acids with respect to Taq DNA polymerase include Asp^{18}, Glu^{117}, Asp^{119}, Asp^{120}, Asp^{142}, and Asp^{144}.

Polymerase mutants can also be made to render the polymerase non-discriminating against non-natural nucleotides such as dideoxynucleotides (see U.S. Pat. No. 5,614, 365). Changes within the O-helix, such as other point mutations, deletions, and insertions, can be made to render the polymerase non-discriminating. By way of example, one Tne DNA polymerase mutant having this property substitutes a non-natural amino acid such as Tyr for Phe730 in the O-helix.

Typically, the 5'-3' exonuclease activity, 3' to 5' exonuclease activity, discriminatory activity and fidelity can be affected by substitution of amino acids typically which have different properties. For example, an acidic amino acid such as Asp may be changed to a basic, neutral or polar but uncharged amino acid such as Lys, Arg, His (basic); Ala, Val, Leu, Ile, Pro, Met, Phe, Trp (neutral); or Gly, Ser, Thr, Cys, Tyr, Asn or Gln (polar but uncharged).Glu may be changed to Asp, Ala, Val Leu, Ile, Pro, Met, Phe, Trp, Gly, Ser, Thr, Cys, Tyr, Asn or Gln.

Preferably, oligonucleotide directed mutagenesis is used to create the mutant polymerases which allows for all possible classes of base pair changes at any determined site along the encoding DNA molecule. In general, this technique involves annealing a oligonucleotide complementary (except for one or more mismatches) to a single stranded nucleotide sequence coding for the DNA polymerase of interest. The mismatched

oligonucleotide is then extended by DNA polymerase, generating a double stranded DNA molecule which contains the desired change in sequence on one strand. The changes in sequence can of course result in the deletion, substitution, or insertion of an amino acid. The double stranded polynucleotide can then be inserted into an appropriate expression vector, and a mutant polypeptide can thus be produced. The above-described oligonucleotide directed mutagenesis can of course be carried out via PCR.

In one embodiment, the non-chimeric mutant *Pfu* DNA polymerases are expressed and purified as described in U.S. Patent No. 5,489,523, hereby incorporated by reference in its entirety.

III. PREPARING DNA POLYMERASE FUSIONS

The DNA polymerase fusion of the invention has at least two polypeptides covalently linked, in which one polypeptide comes from one protein sequence or domain and the other polypeptide comes from another protein sequence or domain. According to the invention, at least one of the domains of the DNA polymerase fusion originates from a wild type or mutant DNA polymerase of the invention. The polypeptides can be linked either directly or via a covalent linker, e.g., an amino acid linker, such as a polyglycine linker, or another type of chemical linker, e.g., a carbohydrate linker, a lipid linker, a fatty acid linker, a polyether linker, e.g., PEG, etc. (See, e.g., Hermanson, Bioconjugate techniques (1996)). The polypeptides forming the fusion polypeptide are typically linked C-terminus to N-terminus, although they can also be linked C-terminus to C-terminus, N-terminus to N-terminus, or N-terminus to C-terminus. One or more polypeptide domains may be inserted at an internal location within a DNA polymerase of the invention. The polypeptides of the fusion protein can be in any order. The term "fusion polypeptide" or "chimera" also refers to conservatively modified variants, polymorphic variants, alleles, mutant, subsequences and interspecies homologues of the polypeptides that make up the fusion protein. Fusion proteins may be produced by covalently linking a chain of amino acids from one protein sequence to a chain of amino acids from another protein sequence, e.g., by preparing a recombinant polynucleotide contiguously encoding the fusion protein. Fusion proteins can comprise 2, 3, 4 or more different chains of amino acids from the same or different species. The different

chains of amino acids in-a fusion protein may be directly spliced together or may be indirectly spliced together via a chemical linking group or an amino acid linking group, which can be about 200 amino acids or more in length, with 1 to 100 amino acids being typical. In some embodiments, proline residues are incorporated into the linker to prevent the formation of significant secondary structural elements by the linker. Linkers can often be flexible amino acid subsequences that are synthesized as part of a recombinant fusion protein. Such flexible linkers are known to persons of skill in the art.

In a preferred embodiment, the DNA polymerase fusion, useful according to the invention, is a thermostable DNA polymerase with reduced DNA polymerization activity or with reduced uracil detection activity. In addition, the DNA polymerase fusion of the invention may or may not have 3'-5' exonuclease activity.

In one embodiment, the component fused to the DNA polymerase is any non-native protein or protein domain fused to the DNA polymerase at the N- or C-terminus or at any internal position. The contribution to the activity of the DNA polymerase from the DNA polymerase fusion partner (that is the second amino acid sequence of the fusion as described herein) includes, but is not limited to, an increase in one or more of the following DNA polymerase activities: processivity, DNA binding, strand displacement activity, polymerase activity, nucleotide binding and recognition, proofreading, fidelity, and salt resistance and/or decreased DNA polymerase activity at room temperature.

A DNA polymerase fusion can be prepared by molecular biology techniques for preparing fusion proteins well known in the art.

Using techniques well known in the art (Sambrook et al., (1989) in: Molecular Cloning, A Laboratory Manual (2nd Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N,Y.), a protein domain of a DNA polymerase can be substituted with a domain from another polymerase which has the desired activity. Methods of preparing a DNA polymerase fusions of the invention are also described in WO 01/92501 A1 and Pavlov et al., 2002, Proc. Natl. Acad. Sci. USA, 99:13510-13515, which are herein incorporated in its entirety.

In one embodiment, the DNA polymerase fusion of the invention comprises a protein domain of one wild type DNA polymerase of the invention that is fused to a protein domain of a different DNA polymerase of the invention containing one or more mutations.

In another preferred embodiment, the DNA polymerase fusion of the invention comprises all of or a part of Pfu or Taq DNA polymerase.

In one embodiment, the DNA polymerase fusion of the invention comprises a *Pfu* DNA polymerase, or part thereof, having reduced DNA polymerization as disclosed in the pending U.S. patent application Serial No.: 10/035,091 (Hogrefe, et al.; filed: December 21, 2001); the pending U.S. patent application Serial No.: 10/079,241 (Hogrefe, et al.; filed February 20, 2002); the pending U.S. patent application Serial No.: 10/208,508 (Hogrefe et al.; filed July 30, 2002); and the pending U.S. patent application Serial No.: 10/227,110 (Hogrefe et al.; filed August 23, 2002), the contents of which are hereby incorporated by reference in their entirety.

In one embodiment, the DNA polymerase fusion of the invention comprises a *Pfu* DNA polymerase, or part thereof, having one or mutations that reduce base analog detection activity as disclosed in the pending U.S. patent application Serial No.: 10/280,962 (Hogrefe, et al.; filed: October 25, 2002) and the pending U.S. patent application Serial No.: 10/298,680 (Hogrefe et al.; filed November 18, 2002) and the pending U.S. patent application Serial No.: 10/324,846 (Borns et al.; filed December 20, 2002), the contents of which are hereby incorporated by reference in their entirety.

In one embodiment, the DNA polymerase fusion of the invention comprises a protein domain of one mutant DNA polymerase of the invention that is fused to a protein domain of a different DNA polymerase of the invention containing one or more mutations.

In one embodiment, the DNA polymerase fusion of the invention comprises a protein domain of one DNA polymerase that replaces an analogous protein domain within another DNA polymerase of the invention. As used herein, two protein domains are said to be "analogous" if they share in common a domain that confers at least one DNA polymerase activity such as processivity, DNA binding, strand displacement activity, nucleotide binding

and recognition, proofreading, e.g. 3'-5' exonuclease activity, fidelity, e.g. 5'-3' exonuclease activity, or salt resistance.

In one embodiment, the DNA polymerase fusion of the invention comprises the helix-hairpin-helix DNA binding motifs from DNA topoisomerase V that increases processivity, salt resistance and thermostability as described in Pavlov et al., 2002, Proc. Natl. Acad. Sci USA, 99:13510-13515.

In another embodiment, the DNA polymerase fusion of the invention comprises the thioredoxin binding domain that enhances the processivity of the DNA polymerase fusion as described in WO 97/29209.

In another embodiment, the DNA polymerase fusion of the invention comprises the archaeal PCNA binding domain fused to *Taq* DNA polymerase or a related eubacterial DNA polymerase. Addition of PCNA to the PCR reaction containing the PCNA binding domain-Taq DNA polymerase chimera results in enhanced processivity of the DNA polymerase fusion and higher yields of PCR amplified DNA (Motz, M., *et al.*, J. Biol. Chem. 2002 May 3; 277 (18); 16179-88).

In another embodiment, the DNA polymerase fusion of the invention comprises the sequence non-specific DNA binding protein Sso7d or Sac7d from (for example, from *Sulfolobus sulfataricus* fused to a DNA polymerase of the invention. The fusion of the DNA binding protein Sso7d or Sac7d to DNA polymerase fusions of the invention, such as *Pfu* or *Taq* DNA polymerase, greatly enhances the processivity of these DNA polymerases as disclosed in WO 01/92501 A1 which is hereby incorporated by reference in its entirety.

The invention contemplates DNA polymerase fusions wherein any of the HhH domains known in the art (see Belova et al., 2001, Proc. Natl. Acad. Sci. USA, 98:6015 and Figure 18) are fused to any of the wildtype or mutant DNA polymerases included herein. The HhH can be fused directly to the N or C terminus or at any internal site of any of the wildtype or mutant DNA polymerases included herein. One of more (for example the H-L or E-L) HhH domains can be used to create a DNA polymerase fusion.

In another embodiment, the DNA polymerase fusion of the invention comprises a *Pfu* DNA polymerase, or part thereof, having reduced 3'-5' exonuclease activity. Methods used to generate 3'-5' exonuclease deficient JDF-3 DNA polymerases including the D141A and E143A mutations are disclosed in the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000), the contents of which are hereby incorporated by reference in their entirety. A person skilled in the art in possession of the teachings of the pending U.S. patent application Serial No.: 09/698,341 (Sorge et al; filed October 27, 2000) would have no difficulty introducing both the corresponding D141A and E143A mutations or other 3'-5' exonuclease mutations into any one of the DNA polymerase fusions of the invention i.e. a DNA polymerase fusion with reduced base analog detection activity or reduced DNA polymerization activity as disclosed herein.

In another embodiment, the DNA polymerase fusion of the invention comprises a DNA polymerase, or part thereof, that lacks both 5' to 3' and 3' to 5' exonuclease activities including, but not limited to, Sequenase (exo$^-$ T7; USB), *Pfu* exo$^-$ (Stratagene), exo$^-$ Vent (New England BioLabs), exo$^-$ DeepVent (New England BioLabs), exo$^-$ Klenow fragment (Stratagene), *Bst* (Bio-Rad), Isotherm (Epicentre), Ladderman (Panvera), KlenTaq1 (Ab Peptides), Stoffel fragment (Perkin-Elmer), ThermoSequenase (USB), and TaqFS (Hoffman-LaRoche), any one of which may be used as the chimeric DNA polymerase fusion of the invention disclosed herein.

In another embodiment, the DNA polymerase fusion of the invention comprises a thermostable DNA polymerase, or part thereof, that has enhanced 3' to 5' exonuclease activity that confers enhanced fidelity to the DNA polymerase fusion of the invention as disclosed in US 5,795,762, the contents of which are hereby incorporated by reference in their entirety.

IV. EXPRESSION OF WILD-TYPE OR MUTANT ENZYMES ACCORDING TO THE INVENTION

Methods known in the art may be applied to express and isolate DNA polymerases of the invention. Many bacterial expression vectors contain sequence elements or combinations of sequence elements allowing high level inducible expression of the protein encoded by a

foreign sequence. For example, as mentioned above, bacteria expressing an integrated inducible form of the T7 RNA polymerase gene may be transformed with an expression vector bearing a mutated DNA polymerase gene linked to the T7 promoter. Induction of the T7 RNA polymerase by addition of an appropriate inducer, for example, isopropyl-β-D-thiogalactopyranoside (IPTG) for a lac-inducible promoter, induces the high level expression of the mutated gene from the T7 promoter.

Appropriate host strains of bacteria may be selected from those available in the art by one of skill in the art. As a non-limiting example, E. coli strain BL-21 is commonly used for expression of exogenous proteins since it is protease deficient relative to other strains of E. coli. BL-21 strains bearing an inducible T7 RNA polymerase gene include WJ56 and ER2566 (Gardner & Jack, 1999, supra). For situations in which codon usage for the particular polymerase gene differs from that normally seen in E. coli genes, there are strains of BL-21 that are modified to carry tRNA genes encoding tRNAs with rarer anticodons (for example, argU, ileY, leuW, and proL tRNA genes), allowing high efficiency expression of cloned protein genes, for example, cloned archaeal enzyme genes (several BL21-CODON PLUSTM cell strains carrying rare-codon tRNAs are available from Stratagene, for example).

There are many methods known to those of skill in the art that are suitable for the purification of a DNA polymerase of the invention. For example, the method of Lawyer et al. (1993, PCR Meth. & App. 2: 275) is well suited for the isolation of DNA polymerases expressed in E. coli, as it was designed originally for the isolation of Taq polymerase. Alternatively, the method of Kong et al. (1993, J. Biol. Chem. 268: 1965, incorporated herein by reference) may be used, which employs a heat denaturation step to destroy host proteins, and two column purification steps (over DEAE-Sepharose and heparin-Sepharose columns) to isolate highly active and approximately 80% pure DNA polymerase. Further, DNA polymerases may be isolated by an ammonium sulfate fractionation, followed by Q Sepharose and DNA cellulose columns, or by adsorption of contaminants on a HiTrap Q column, followed by gradient elution from a HiTrap heparin column.

V. BLENDS OF FUSION AND NON-FUSION DNA POLYMERASES

A DNA polymerase fusion blend formulation, according to the invention, can include at least one DNA polymerase fusion and: (1) a proofreading or a non-proofreading non-chimeric DNA polymerase; or (2) a proofreading plus non-proofreading, non-proofreading plus non-proofreading or a proofreading plus proofreading non-fusion DNA polymerase blend, *e.g.*, *Pfu*, *Taq*, *Pfu/Taq*, *Pfu*/exo-*Pfu*, *Taq*/exo-*Pfu*, *Pfu*/JDF3, or any of these combinations with pol-*Pfu* (*Pfu* G387P). The ratio of DNA polymerase enzymes in a "blend" comprising one fusion and one non-fusion polymerase is in the range of 1:1-1:5-5:1, or 1:1-1:10-10:1, or 1:1-1:25-25:1 or 1:1-1:100-100:1. For embodiments wherein a "blend" comprises one DNA polymerase fusion and two non-fusion polymerases the ratio of the first non-fusion DNA polymerase to the second non-fusion DNA polymerase is in the range of 1:1-1:5-5:1, or 1:1-1:10-10:1, or 1:1-1:25-25:1 or 1:1-1:100-100:1. The formulation of the invention has no limitations on the ratios of the individual components.

In one embodiment, the blend formulation of the invention is 2.5U *Pfu* / 0.25U chimeric *Pfu*.

The wild type DNA polymerase that is blended with the DNA polymerase fusion can be any native or cloned DNA polymerase having native levels of polymerase activity and proofreading activity and preferably is thermostable such as *Pfu* or *Taq*. The DNA polymerase fusion and wild type DNA polymerase are blended in the ratio range described above and can be mixed with any replication accessory factor or PCR enhancing additives, *e.g.*, *Pfu* dUTPase (PEF), PCNA, RPA, ssb, antibodies, DMSO, betaine, or 3'-5' exonuclease (e.g., *Pfu* G387P).

The mutant DNA polymerase that is blended with the DNA polymerase fusion of the invention is any DNA polymerase having introduced mutations and/or truncations that generates a DNA polymerase with an activity that is distinct from a wild type DNA polymerase. The mutant could have any amount of polymerase and/or proofreading activity. Specific examples of useful mutations or truncations include, but are not limited to, V93R,K,E, or D in *Pfu* DNA polymerase, which confer uracil insensitivity, D141A / E143A

in *Pfu* DNA polymerase, which eliminates 3'-5' exonuclease activity, and the N-terminal truncation of *Taq* that eliminates 5'-3' exonuclease activity (Klen*Taq*).

The invention further provides for mutant V93R, V93E, V93D, V93K or V93N non-fusion Pfu DNA polymerases that contain one or more additional mutations with improved reverse transcriptase activity.

The invention provides for a blend wherein the ratio of DNA polymerase fusion to non-fusion DNA polymerase is in the ratio range of 1:1-1:5-5:1, or 1:1-1:10-10:1, or 1:1-1:25-25:1 or 1:1-1:100-100:1. The invention contemplates a blend comprising a mixture of a DNA polymerase fusion and more than one non-fusion DNA polymerase. For a blend comprising a DNA polymerase fusion in combination with two non-fusion DNA polymerases, the ratio range of the first non-fusion DNA polymerases to the second non-fusion DNA polymerase is 1:1-1:5-5:1, or 1:1-1:10-10:1, or 1:1-1:25-25:1 or 1:1-1:100-100:1.

VI. APPLICATIONS OF THE SUBJECT INVENTION

The invention provides for methods of using polymerase fusions of the invention at high pH as defined herein.

A high pH buffer useful according to the invention includes but is not limited to a standard PCR reaction buffer like cloned *Pfu* reaction buffer (described in Example 3) wherein the buffering component is at a high pH (i.e. 9.3-14). The buffering component used in the following examples is 30 mM Tris [Tris(hydroxymethyl) aminomethane] at a pH of 10.0 or 11.8. The pH of the buffering component in standard PCR reaction buffers is from 8.3 – 8.8. The buffering component is used at a concentration from 1mM to 1M in the final PCR reaction and may be any pH from 9.5-14. The buffering component of the present invention includes, but is not limited to, Tris, Tricine, bicine, Bis-Tris, CAPS, EPPS, HEPES, MES, MOPS, PIPES, TAPS and TES.

In one aspect, the invention provides a method for DNA synthesis using the compositions of the subject invention. Typically, synthesis of a polynucleotide requires a synthesis primer, a synthesis template, polynucleotide precursors for incorporation into the newly synthesized polynucleotide, (e.g. dATP, dCTP, dGTP, dTTP), and the like. Detailed

methods for carrying out polynucleotide synthesis are well known to the person of ordinary skill in the art and can be found, for example, in Molecular Cloning second edition, Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

A. APPLICATION IN AMPLIFICATION REACTIONS

"Polymerase chain reaction" or "PCR" refers to an in vitro method for amplifying a specific polynucleotide template sequence. The technique of PCR is described in numerous publications, including, PCR: A Practical Approach, M. J. McPherson, et al., IRL Press (1991), PCR Protocols: A Guide to Methods and Applications, by Innis, et al., Academic Press (1990), and PCR Technology: Principals and Applications for DNA Amplification, H. A. Erlich, Stockton Press (1989). PCR is also described in many U.S. Patents, including U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; 4,965,188; 4,889,818; 5,075,216; 5,079,352; 5,104,792; 5,023,171; 5,091,310; and 5,066,584, each of which is herein incorporated by reference.

For ease of understanding the advantages provided by the present invention, a summary of PCR is provided. The PCR reaction involves a repetitive series of temperature cycles and is typically performed in a volume of 50-100 μl. The reaction mix comprises dNTPs (each of the four deoxynucleotides dATP, dCTP, dGTP, and dTTP), primers, buffers, DNA polymerase, and polynucleotide template. PCR requires two primers that hybridize with the double-stranded target polynucleotide sequence to be amplified. In PCR, this double-stranded target sequence is denatured and one primer is annealed to each strand of the denatured target. The primers anneal to the target polynucleotide at sites removed from one another and in orientations such that the extension product of one primer, when separated from its complement, can hybridize to the other primer. Once a given primer hybridizes to the target sequence, the primer is extended by the action of a DNA polymerase. The extension product is then denatured from the target sequence, and the process is repeated.

In successive cycles of this process, the extension products produced in earlier cycles serve as templates for DNA synthesis. Beginning in the second cycle, the product of amplification begins to accumulate at a logarithmic rate. The amplification product is a discrete double-stranded DNA molecule comprising: a first strand which contains the

sequence of the first primer, eventually followed by the sequence complementary to the second primer, and a second strand which is complementary to the first strand.

Due to the enormous amplification possible with the PCR process, small levels of DNA carryover from samples with high DNA levels, positive control templates or from previous amplifications can result in PCR product, even in the absence of purposefully added template DNA. If possible, all reaction mixes are set up in an area separate from PCR product analysis and sample preparation. The use of dedicated or disposable vessels, solutions, and pipettes (preferably positive displacement pipettes) for RNA/DNA preparation, reaction mixing, and sample analysis will minimize cross contamination. See also Higuchi and Kwok, 1989, Nature, 339:237-238 and Kwok, and Orrego, in: Innis et al. eds., 1990, PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc., San Diego, Calif., which are incorporated herein by reference.

The enzymes provided herein are also useful for dUTP/UNG cleanup methods that require PCR enzymes that incorporate dUTP (Longo et al., Supra).

1. THERMOSTABLE ENZYMES

For PCR amplifications, the enzymes used in the invention are preferably thermostable. As used herein, "thermostable" refers to an enzyme which is stable to heat, is heat resistant, and functions at high temperatures, e.g., 50 to 90°C. The thermostable enzyme according to the present invention must satisfy a single criterion to be effective for the amplification reaction, i.e., the enzyme must not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded polynucleotides. By "irreversible denaturation" as used in this connection, is meant a process bringing a permanent and complete loss of enzymatic activity. The heating conditions necessary for denaturation will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the polynucleotides being denatured, but typically range from 85°C, for shorter polynucleotides, to 105°C for a time depending mainly on the temperature and the polynucleotide length, typically from 0.25 minutes for shorter polynucleotides, to 4.0 minutes for longer pieces of DNA. Higher temperatures may be tolerated as the buffer salt concentration and/or GC composition of the polynucleotide is

increased. Preferably, the enzyme will not become irreversibly denatured at 90 to 100°C. An enzyme that does not become irreversibly denatured, according to the invention, retains at least 10%, or at least 25%, or at least 50% or more function or activity during the amplification reaction.

2. PCR REACTION MIXTURE

In addition to the subject enzyme mixture, one of average skill in the art may also employ other PCR parameters to increase the fidelity of synthesis/amplification reaction. It has been reported that PCR fidelity may be affected by factors such as changes in dNTP concentration, units of enzyme used per reaction, pH, and the ratio of Mg^{2+} to dNTPs present in the reaction (Mattila et al., 1991, supra).

Mg^{2+} concentration affects the annealing of the oligonucleotide primers to the template DNA by stabilizing the primer-template interaction, it also stabilizes the replication complex of polymerase with template-primer. It can therefore also increase non-specific annealing and produce undesirable PCR products (gives multiple bands in gel). When non-specific amplification occurs, the Mg^{2+} concentration may need to be lowered or EDTA can be added to chelate Mg^{2+} to increase the accuracy and specificity of the amplification.

Other divalent cations such as Mn^{2+}, or Co^{2+} can also affect DNA polymerization. Suitable cations for each DNA polymerase are known in the art (e.g., in DNA Replication 2nd edition, supra). Divalent cation is supplied in the form of a salt such $MgCl_2$, $Mg(OAc)_2$, $MgSO_4$, $MnCl_2$, $Mn(OAc)_2$, or $MnSO_4$. Usable cation concentrations in a Tris-HCl buffer are for $MnCl_2$ from 0.5 to 7 mM, preferably, between 0.5 and 2 mM, and for $MgCl_2$ from 0.5 to 10 mM. Usable cation concentrations in a Bicine/KOAc buffer are from 1 to 20 mM for $Mn(OAc)_2$, preferably between 2 and 5 mM.

Monovalent cation required by DNA polymerase may be supplied by the potassium, sodium, ammonium, or lithium salts of either chloride or acetate. For KCl, the concentration is between 1 and 200 mM, preferably the concentration is between 40 and 100 mM, although the optimum concentration may vary depending on the polymerase used in the reaction.

Deoxyribonucleotide triphosphates (dNTPs) are added as solutions of the salts of dATP, dCTP, dGTP, dUTP, and dTTP, such as disodium or lithium salts. In the present methods, a final concentration in the range of 1 μM to 2 mM each is suitable, and 100-600 μM is preferable, although the optimal concentration of the nucleotides may vary in the PCR reaction depending on the total dNTP and divalent metal ion concentration, and on the buffer, salts, particular primers, and template. For longer products, i.e., greater than 1500 bp, 500 μM each dNTP may be preferred when using a Tris-HCl buffer.

dNTPs chelate divalent cations, therefore amount of divalent cations used may need to be changed according to the dNTP concentration in the reaction. Excessive amount of dNTPs (e.g., larger than 1.5 mM) can increase the error rate and possibly inhibit DNA polymerases. Lowering the dNTP (e.g., to 10-50 μM) may therefore reduce error rate. PCR reaction for amplifying larger size template may need more dNTPs.

The PCR reaction buffer is a standard PCR reaction buffer like cloned *Pfu* reaction buffer but with a buffering component at a high pH (i.e. 9.1-14). One suitable buffering component is 30 mM Tris [Tris(hydroxymethyl) aminomethane] at a pH of 10.0 or 11.8. The pH of the buffering component in standard PCR reaction buffers is from 8.3 – 8.8. The buffering component is used at a concentration from 1mM to 1M in the final PCR reaction at a pH from 9.1-14. A buffering component useful in this invention includes, but is not limited to, Tris, Tricine, bicine, Bis-Tris, CAPS, EPPS, HEPES, MES, MOPS, PIPES, TAPS and TES.

PCR is a very powerful tool for DNA amplification and therefore very little template DNA is needed. However, in some embodiments, to reduce the likelihood of error, a higher DNA concentration may be used, though too many templates may increase the amount of contaminants and reduce efficiency.

Usually, up to 3 μM of primers may be used, but high primer to template ratio can result in non-specific amplification and primer-dimer formation. Therefore it is usually necessary to check primer sequences to avoid primer-dimer formation.

The invention provides for Pfu V93R, V93E, V93K , V93D , or V93N fusion or non-fusion DNA polymerases with reduced uracil detection activity that enhance PCR of GC rich DNA templates by minimizing the effect of cytosine deamination in the template and by allowing the use of higher denaturation times and denaturation temperatures.

3. CYCLING PARAMETERS

Denaturation time may be increased if template GC content is high. Higher annealing temperature may be needed for primers with high GC content or longer primers. Gradient PCR is a useful way of determining the annealing temperature. Extension time should be extended for larger PCR product amplifications. However, extension time may need to be reduced whenever possible to limit damage to enzyme.

The number of cycles can be increased if the number of template DNA is very low, and decreased if high amount of template DNA is used.

4. PCR ENHANCING FACTORS AND ADDITIVES

PCR enhancing factors may also be used to improve efficiency of the amplification. As used herein, a "PCR enhancing factor" or a "Polymerase Enhancing Factor" (PEF) refers to a complex or protein possessing polynucleotide polymerase enhancing activity (Hogrefe et al., 1997, Strategies 10::93-96; and U.S. Patent No. 6,183,997, both of which are hereby incorporated by references). For *Pfu* DNA polymerase, PEF comprises either P45 in native form (as a complex of P50 and P45) or as a recombinant protein. In the native complex of *Pfu* P50 and P45, only P45 exhibits PCR enhancing activity. The P50 protein is similar in structure to a bacterial flavoprotein. The P45 protein is similar in structure to dCTP deaminase and dUTPase, but it functions only as a dUTPase converting dUTP to dUMP and pyrophosphate. PEF, according to the present invention, can also be selected from the group consisting of: an isolated or purified naturally occurring polymerase enhancing protein obtained from an archeabacteria source (e.g., *Pyrococcus furiosus*); a wholly or partially synthetic protein having the same amino acid sequence as *Pfu* P45, or analogs thereof possessing polymerase enhancing activity; polymerase-enhancing mixtures of one or more of said naturally occurring or wholly or partially synthetic proteins; polymerase-enhancing

protein complexes of one or more of said naturally occurring or wholly or partially synthetic proteins; or polymerase-enhancing partially purified cell extracts containing one or more of said naturally occurring proteins (U.S. Patent No. 6,183,997, supra). The PCR enhancing activity of PEF is defined by means well known in the art. The unit definition for PEF is based on the dUTPase activity of PEF (P45), which is determined by monitoring the production of pyrophosphate (PPi) from dUTP. For example, PEF is incubated with dUTP (10mM dUTP in 1x cloned *Pfu* PCR buffer) during which time PEF hydrolyzes dUTP to dUMP and PPi. The amount of PPi formed is quantitated using a coupled enzymatic assay system that is commercially available from Sigma (#P7275). One unit of activity is functionally defined as 4.0 nmole of PPi formed per hour (at 85°C).

Other PCR additives may also affect the accuracy and specificity of PCR reactions. EDTA less than 0.5 mM may be present in the amplification reaction mix. Detergents such as Tween-20™ and Nonidet™ P-40 are present in the enzyme dilution buffers. A final concentration of non-ionic detergent approximately 0.1% or less is appropriate, however, 0.01-0.05% is preferred and will not interfere with polymerase activity. Similarly, glycerol is often present in enzyme preparations and is generally diluted to a concentration of 1-20% in the reaction mix. Glycerol (5-10%), formamide (1-5%) or DMSO (2-10%) can be added in PCR for template DNA with high GC content or long length (e.g., > 1kb). These additives change the Tm (melting temperature) of primer-template hybridization reaction and the thermostability of polymerase enzyme. BSA (up to 0.8 μg/μl) can improve efficiency of PCR reaction. Betaine (0.5-2M) is also useful for PCR over high GC content and long fragments of DNA. Tetramethylammonium chloride (TMAC, >50mM), Tetraethylammonium chloride (TEAC), and Trimethlamine N-oxide (TMANO) may also be used. Test PCR reactions may be performed to determine optimum concentrations of each additive mentioned above.

The invention provides for additives including, but not limited to antibodies (for hot start PCR) and ssb (single strand DNA binding protein; higher specificity). The invention also contemplates mutant archael DNA polymerases in combination with accessory factors, for example as described in U.S. 6,333,158, and WO 01/09347 A2, hereby incorporated by reference in its entirety.

Various specific PCR amplification applications are available in the art (for reviews, see for example, Erlich, 1999, Rev Immunogenet., 1:127-34; Prediger 2001, Methods Mol. Biol. 160:49-63; Jurecic et al., 2000, Curr. Opin. Microbiol. 3:316-21; Triglia, 2000, Methods Mol. Biol. 130:79-83; MaClelland et al., 1994, PCR Methods Appl. 4:S66-81; Abramson and Myers, 1993, Current Opinion in Biotechnology 4:41-47; each of which is incorporated herein by references).

The subject invention can be used in PCR applications including, but not limited to, i) hot-start PCR which reduces non-specific amplification; ii) touch-down PCR which starts at high annealing temperature, then decreases annealing temperature in steps to reduce non-specific PCR product; iii) nested PCR which synthesizes more reliable product using an outer set of primers and an inner set of primers; iv) PCR for amplification of regions flanking a known sequence; (in this method, DNA is digested, the desired fragment is circularized by ligation, then PCR using primer complementary to the known sequence extending outwards; v) AP-PCR (arbitrary primed)/RAPD (random amplified polymorphic DNA); these methods create genomic fingerprints from species with little-known target sequences by amplifying using arbitrary oligonucleotides; vi) RT-PCR which uses RNA-directed DNA polymerase (e.g., reverse transcriptase) to synthesize cDNAs which is then used for PCR. This method is extremely sensitive for detecting the expression of a specific sequence in a tissue or cell. It may also be used to quantify mRNA transcripts; vii) RACE (rapid amplification of cDNA ends). This is used where information about DNA/protein sequence is limited. The method amplifies 3' or 5' ends of cDNAs generating fragments of cDNA with only one specific primer each (plus one adaptor primer). Overlapping RACE products can then be combined to produce full length cDNA; viii) DD-PCR (differential display PCR) which is used to identify differentially expressed genes in different tissues. First step in DD-PCR involves RT-PCR, then amplification is performed using short, intentionally nonspecific primers; ix) Multiplex-PCR in which two or more unique targets of DNA sequences in the same specimen are amplified simultaneously. One DNA sequence can be used as a control to verify the quality of PCR; x) Q/C-PCR (Quantitative comparative) which uses an internal control DNA sequence (but of a different size) which competes with the target DNA (competitive PCR) for the same set of primers; xi) Recusive PCR which is used to synthesize genes. Oligonucleotides used in this method are complementary to stretches of a gene (>80 bases),

alternately to the sense and to the antisense strands with ends overlapping (~20 bases); xii) Asymmetric PCR; xiii) In Situ PCR; xiv) Site-directed PCR Mutagenesis.

It should be understood that this invention is not limited to any particular amplification system. As other systems are developed, those systems may benefit by practice of this invention.

B. APPLICATION IN DIRECT CLONING OF PCR AMPLIFIED PRODUCT

It is understood that the amplified product produced using the subject enzyme can be cloned by any method known in the art. In one embodiment, the invention provides a composition which allows direct cloning of PCR amplified product.

The most common method for cloning PCR products involves incorporation of flanking restriction sites onto the ends of primer molecules. The PCR cycling is carried out and the amplified DNA is then purified, restricted with an appropriate endonuclease(s) and ligated to a compatible vector preparation.

A method for directly cloning PCR products eliminates the need for preparing primers having restriction recognition sequences and it would eliminate the need for a restriction step to prepare the PCR product for cloning. Additionally, such method would preferably allow cloning PCR products directly without an intervening purification step.

U.S. Patent Nos. 5,827,657 and 5,487,993 (hereby incorporated by their entirety) disclose methods for direct cloning of PCR products using a DNA polymerase which takes advantage of the single 3'-deoxy-adenosine monophosphate (dAMP) residues attached to the 3' termini of PCR generated nucleic acids. Vectors are prepared with recognition sequences that afford single 3'-terminal deoxy-thymidine monophosphate (dTMP) residues upon reaction with a suitable restriction enzyme. Thus, PCR generated copies of genes can be directly cloned into the vectors without a need for preparing primers having suitable restriction sites therein.

Taq DNA polymerase exhibits terminal transferase activity that adds a single dATP to the 3' ends of PCR products in the absence of template. This activity is the basis for the TA

cloning method in which PCR products amplified with Taq are directly ligated into vectors containing single 3'dT overhangs. *Pfu* DNA polymerase, on the other hand, lacks terminal transferase activity, and thus produces blunt-ended PCR products that are efficiently cloned into blunt-ended vectors. The invention also encompasses an Easy A composition that contains of a blend of Taq (5U/ul), recombinant PEF (4U/ul), and Pfu G387P(40ng/ul) as disclosed in the pending U.S. patent application Serial No.: 10/035,091 (Hogrefe, et al.; filed: December 21, 2001); the pending U.S. patent application Serial No.: 10/079,241 (Hogrefe, et al.; filed February 20, 2002); the pending U.S. patent application Serial No.: 10/208,508 (Hogrefe et al.; filed July 30, 2002); and the pending U.S. patent application Serial No.: 10/227,110 (Hogrefe et al.; filed August 23, 2002), the contents of which are hereby incorporated in their entirety. With cloned archaeal DNA polymerase with reduced base analog detection activity at 2.5U/ul i.e. ~20-50ng per ul, the ratio of Taq:Pfu is preferably 1:1 or more preferably 2:1 or more.

In one embodiment, the invention provides for a PCR product, generated in the presence of a DNA polymerase fusion at high pH, that is subsequently incubated with Taq DNA polymerase in the presence of dATP at 72°C for 15-30 minutes. Addition of 3'-dAMP to the ends of the amplified DNA product then permits cloning into TA cloning vectors according to methods that are well known to a person skilled in the art.

C. APPLICATION IN DNA SEQUENCING

The invention further provides for dideoxynucleotide DNA sequencing methods using thermostable DNA polymerase fusions to catalyze the primer extension reactions at high pH. Methods for dideoxynucleotide DNA sequencing are well known in the art and are disclosed in U.S. Patent Nos. 5,075,216, 4,795,699 and 5,885,813, the contents of which are hereby incorporated in their entirety. The invention encompasses DNA polymerase fusions comprising exo-Pfu (for example D141A/E143A double mutant) or the JDF3 P410L/A485T mutant with reduced ddNTP discrimination.

D. APPLICATION IN MUTAGENESIS

The DNA polymerase fusions of the invention also provide enhanced efficacy for PCR-based or linear amplification-based mutagenesis. The invention therefore provides for DNA polymerase fusions for site-directed mutagenesis at high pH and their incorporation into commercially available kits, for example, QuikChange Site-directed Mutagenesis, QuikChange Multi-Site-Directed Mutagenesis (Stratagene). Site-directed mutagenesis methods and reagents are disclosed in the pending U.S. Patent Application No. 10/198,449 (Hogrefe et al.; filed July 18, 2002), the contents of which are hereby incorporated in its entirety. The invention also encompasses Mutazyme (exo$^-$Pfu in combination with PEF, GeneMorph Kit). The GeneMorph kits are disclosed in the pending U.S. Patent Application No.: 10/154,206 (filed May 23, 2002), the contents of which are hereby incorporated in its entirety.

The DNA polymerase fusions described herein are used in the same way as conventional DNA polymerase/ DNA polymerase formulations and can be used at high pH in any primer extension application, including PCR, to produce high product yields with shortened extension times. Amplification of genomic targets, in particular, which typically require extension times of 1-2 min./kb and take hours to amplify, is greatly facilitated by the disclosed invention because extension times are reduced to 5-30sec./kb, or shorter, with the DNA polymerase fusions described herein (see Example 3).

Other applications of the present invention include RT-PCR, site-directed mutagenesis and random mutagenesis. The DNA polymerase fusions of the invention used in all of these applications increase length capability, shorten reaction times and greatly improve overall performance in all standard protocols (see Example 3).

A DNA polymerase fusion with proofreading activity (3'-5' exonuclease activity) is useful for high fidelity PCR: A DNA polymerase fusion that is useful for high fidelity PCR will demonstrate an increase of $\geq$ 10% 3'-5' exonuclease activity and PCR fidelity, and accuracy of incorporation as compared to a corresponding non-fusion polymerase (with 3'-5' exonuclease activity) alone using a complex genomic and / or plasmid template.

A DNA polymerase fusion with higher misinsertion and / or mispair extension frequency is useful for PCR random mutagenesis. A DNA polymerase fusion that is useful for PCR random mutagenesis preferably demonstrates an increase of $\geq$10% of the mutagenic properties or changes in mutational spectra as compared to a corresponding non-fusion polymerase for plasmid template.

By "mutagenic properties" is meant mutation rate and the overall number of mutation instances per kb of amplicon.

By "mutational spectra" is meant the number of transition and transversion mutations. "Mutational spectra" also encompasses the ratio of transitions to transversions. Preferably the ratio of transitions to transversion is 1:1.

All of the DNA polymerase fusions contemplated herein are useful for PCR and RT-PCR:

DNA polymerase fusions with proofreading activity that are used for PCR amplification and linear amplification are useful for Site Directed Mutagenesis.

DNA polymerase fusions that lack 3'-5' exonuclease activity are useful for sequencing applications. A DNA polymerase fusion useful for sequencing will demonstrate one or more of shorter extension times, higher efficiency, higher specificity, higher fidelity (more accurate incorporation), and higher processivity (an increase of $\geq$10% above the non-chimeric component of the blend for sequencing template). DNA polymerase fusions that lack 3'-5' exonuclease activity are also useful for random mutagenesis.

KITS

The invention herein also contemplates a kit format which comprises a package unit having one or more containers of the subject composition and in some embodiments including containers of various reagents used for polynucleotide synthesis, including synthesis in PCR. The kit may also contain one or more of the following items: polynucleotide precursors, primers, buffers (preferably a high pH buffer), instructions, and controls. Kits may include containers of reagents mixed together in suitable proportions for performing the methods in

accordance with the invention. Reagent containers preferably contain reagents in unit quantities that obviate measuring steps when performing the subject methods.

The invention contemplates a kit comprising a DNA polymerase fusion and a high pH buffer according to the invention, PCR enhancing reagents and reagents for PCR amplification, DNA sequencing or mutagenesis.

A kit for sequencing DNA will comprise a number of container means. A first container means may, for example, comprise a substantially purified sample of the polymerases of the invention. A second container means may comprise one or a number of types of nucleotides needed to synthesize a DNA molecule complementary to DNA template. A third container means may comprise one or a number of different types of terminators (such as dideoxynucleoside triphosphates). A fourth container means may comprise pyrophosphatase. In addition to the above container means, additional container means may be included in the kit which comprise one or a number of primers and/or a suitable sequencing buffer, preferably a high pH buffer.

A kit used for amplifying or synthesis of nucleic acids will comprise, for example, a first container means comprising a substantially pure polymerase fusion of the invention and one or a number of additional container means which comprise a single type of nucleotide or mixtures of nucleotides, and/or a high pH buffer.

Various primers may be included in a kit as well as a suitable amplification or synthesis buffers.

When desired, the kit of the present invention may also include container means which comprise detectably labeled nucleotides which may be used during the synthesis or sequencing of a nucleic acid molecule. One of a number of labels may be used to detect such nucleotides. Illustrative labels include, but are not limited to, radioactive isotopes, fluorescent labels, chemiluminescent labels, bioluminescent labels and enzyme labels.

Having now generally described the invention, the same will be more readily understood through reference to the following Examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLES

Example 1.

Construction of DNA Polymerase Fusions

A chimera is made by combining the domains of different DNA polymerases, for example, the insertion of the thioredoxin processivity factor binding domain of bacteriophage T7 DNA polymerase into the homologous site in *E. coli* DNA polymerase I. This facilitates a substantial increase in the processivity of the chimeric *E. coli* DNA polymerase I in the presence of thioredoxin. (Bedford, E., *et al.*, PNAS, USA vol. 94, pp. 479-484, Jan. 1997 Biochem.). Another illustration of this strategy is the addition of an archaeal PCNA binding domain to *Taq* DNA polymerase. PCNA is then added to the PCR reaction with the *Taq* chimera to enhance processivity and generate higher yields (Motz, M., *et al.*, J. Biol. Chem. 2002 May 3; 277 (18); 16179-88).

A chimeric DNA polymerase is also generated by combining elements (protein or domain) of a double stranded DNA binding protein with a DNA polymerase. The helix-hairpin-helix DNA binding motifs from DNA topoisomerase V have been added to the NH(2) terminus or COOH terminus of *Taq* DNA polymerase, Stoffel fragment of *Taq* DNA polymerase or *Pfu* DNA polymerase. The resulting chimeras have increased processivity, salt tolerance, and thermostability (Pavlov, AR., *et al.* PNAS USA 2002, Oct. 15; 99 (21); 13510-5). Another example is the fusion of DNA polymerase with the sequence non-specific DNA binding protein Sso7d or Sac7d from *Sulfolobus sulfataricus*, or an archaeal PCNA DNA binding domain. This strategy is used to enhance the processivity of *Pfu* or *Taq* DNA polymerase (WO 01/92501 A1).

DNA polymerases of the invention including but not limited to Pfu fusion proteins are purified as described in PCT/US01 17492 or Pavlov et al., supra.

Example 2.

Chimeric DNA polymerase blend formulations.

A chimeric DNA polymerase blend formulation is comprised of a chimeric DNA polymerase and: (1) a proofreading or a non-proofreading DNA polymerase; or (2) a

proofreading plus non-proofreading, non-proofreading plus non-proofreading or a proofreading plus proofreading DNA polymerase blend, *e.g.*, *Pfu*, *Taq*, *Pfu/Taq*, *Pfu*/exo-*Pfu*, *Taq*/exo-*Pfu*, *Pfu*/JDF3, or any of these combinations with pol-*Pfu* (*Pfu* G387P). A specific non limiting example of a blend formulation is 2.5U *Pfu* / 0.25U chimeric *Pfu*. A chimeric DNA blend comprises a chimeric DNA polymerase in combination with at least one wild type and/or at least one mutant DNA polymerase (as defined herein).

The wild type DNA polymerase that is blended with the DNA polymerase chimera is any native or cloned DNA polymerase having native levels of polymerase activity, proofreading activity and is preferably thermostable like *Pfu* or *Taq*. The chimeric DNA polymerase and wt DNA polymerase are blended (for example in any ratio described herein) and mixed with any replication accessory factor (a protein that enhances DNA synthesis) or PCR enhancing additives, *e.g.*, *Pfu* dUTPase (PEF), PCNA, RPA, ssb, antibodies, DMSO, betaine, or 3'-5' exonuclease (e.g., *Pfu* G387P). Specific non-limiting examples of commercially useful mutations or truncations are V93R,K,E,D in *Pfu*, which confer uracil insensitivity, D141A / E143A in *Pfu*, which eliminates 3'-5' exonuclease activity, and the N-terminal truncation of *Taq* to eliminate 5'-3' exonuclease activity(Klen*Taq*). The chimeric DNA polymerase and mutant DNA polymerase are blended in any ratio and mixed with any replication accessory factor or PCR additives. The DNA polymerase formulation is any mixture of wt, wt and mutant, mutant and mutant DNA polymerases. The chimeric DNA polymerase and DNA polymerase formulation are blended in any ratio and mixed with any replication accessory factor or PCR additives.

High pH PCR reaction buffer.

A high pH PCR reaction buffer is formulated at a 10X concentration and used in PCR reactions at a final 1X concentration, which is standard for most commercially produced PCR reaction buffers. A 10X buffer formulation useful according to the invention is: 300mM Tris pH 10.0 or pH 11.8; 100mM KCl; 100mM Ammonium Sulfate; 20mM Magnesium Sulfate; 1% Trition X-100; 1mg / ml nuclease-free bovine serum albumin (BSA). This formulation is in no way a limitation of the components or concentrations of components used for the invention. The components of the buffer, other than the buffering component, are varied

depending on the requirements for the maximal activity of a specific DNA polymerase or DNA polymerase blend.

Example 3:

PCR Amplification with a chimeric *Pfu* DNA Polymerase or with DNA polymerase blends Containing a Chimeric *Pfu* DNA polymerase

PCR reaction conditions

PCR reactions were conducted under standard conditions in 1X cloned *Pfu* PCR buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM Mg SO_4, 0.1% Triton X-100, and 100μg/ml BSA) except that 1). The Tris component was at pH 10.0 or 11.8 and at a final concentration of 30mM and 2). The mixture contained 0.25-1.3U *Pfu-Sso7d* chimeric DNA polymerase (sequence provided herein and 10 01/92501, incorporated by reference in its entirety) or chimeric DNA polymerase blends composed of 0.25U *Pfu-Sso7d* and either 2.5U or 5.0U *Pfu* DNA polymerase. All PCR reactions contained 2U / 50μl cloned *Pyrococcus furiosus* dUTPase (PEF). For all genomic targets 0.9-6.0kb in length, PCR reactions contained 100ng of human genomic DNA, 300μM each dNTP, and 100ng of each primer. For the 19kb genomic target, PCR reactions contained 250ng of human genomic DNA, 500μM each dNTP, and 200ng of each primer.

Cycling Conditions and primer sequences:

Target size (kb)	Target gene	Cycling Parameters

0.9	Hα1AT	(1 cycle) 95°C 2 min (30 cycles) 95°C 5 sec, 58°C 5 sec, 72°C 1 sec or 5 sec. (1 cycle) 72°C 2 min
2.6	Hα1AT	(1 cycle) 95°C 2 min (30 cycles) 95°C 20 sec, 58°C 20 sec, 72°C 5 sec or 1 min 30 sec. (1 cycle) 72°C 3 min
6	β globin	(1 cycle) 95°C 2 min (30 cycles)95°C 30 sec, 58°C 30 sec, 72°C 1 min or 1min 30 sec. (1 cycle) 72°C 5 min
19	β globin	(one cycle) 92°C 2 min (10 cycles) 92°C 10 sec, 63°C 30 sec, 68°C 9.5 min (20 cycles) 92°C 10 sec, 63°C 30 sec, 68°C 9.5 min (plus 10 sec/cycle) (one cycle) 68°C 7 min
Primer size (bp)	Target	Primer sequence
30	Hα1AT 0.9kb	F-5'-AGA.GCT.TGA.GGA.GAG.CAG.GAA.AGG.TGG.AAC-3'
30	Hα1AT 0.9kb	R-5'-GGG.AGG.GGA.GGT.ACA.GGG.TTG.AGG.CTA.GTG-3'
30	Hα1AT 2.6kb	F-5'-AGA.GCT.TGA.GGA.GAG.CAG.GAA.AGG.TGG.AAC-3'
24	Hα1AT 2.6kb	R-5'-TGC.AGA.GCG.ATT.ATT.CAG.GAA.TGC-3'

30	β globin 6.0kb	F-5'-ACA.AGG.GCT.ACT.GGT.TGC.CGA.TTT.TTA.TTG-3'
27	β globin 6.0kb	R-5'-GGG.ACT.GGC.CTC.AGA.GGA.AAC.TTC.AGG-3'
30	β globin 19kb	F-5'-ACA.AGG.GCT.ACT.GGT.TGC.CGA.TTT.TTA.TTG-3'
28	β globin 19kb	R-5'-CCT.GCA.TTT.GTG.GGG.TGA.ATT.CCT.TGC.C-3'

Effect of buffer pH on PCR amplification

To demonstrate the effect of pH on PCR reactions with chimeric *Pfu*-Sso7d DNA polymerase, PCR reactions were prepared using 1X *Pfu* reaction buffer wherein the pH of the Tris component was titrated from pH 5.0-12.0 (figures # 1 & 2). *Pfu*-Sso7d / *Pfu* Turbo blends (0.25U *Pfu*-Sso7d + 2.5U or 5.0U *Pfu* Turbo) were used to amplify a 6kb human beta globin genomic target with an extension time of 15 seconds per kb. *Pfu* Turbo alone cannot amplify this target at 15 seconds per kb. Amplification is only achieved with the contribution of the more processive *Pfu*-Sso7d. Amplification appears at pH 8.5 and is strongest between pH 10.0-12.0, demonstrating the enhancing effect of high pH on the chimeric *Pfu*-Sso7d DNA polymerase (figures 1 & 2).

To demonstrate the enhancing effect of a high pH PCR reaction buffer for the PCR amplification of long genomic targets, a 19kb fragment of human beta globin was amplified using *Pfu*-Sso7d / *Pfu* Turbo blend with an extension time of 30 seconds per kb. Amplification of this target with an extension time of 30 seconds per kb can only be achieved with the contribution of the more processive *Pfu*-Sso7d chimeric DNA polymerase component of the blend. PCR amplification in the pH 10.0 and pH 11.8 reaction buffers was compared to amplification in 1.5X cloned *Pfu* reaction buffer, which is the optimal PCR reaction buffer condition for *Pfu* Turbo (Strategies: Vol. 12, #4; "High fidelity PCR of genomic targets up to 19kb"). PCR reactions using the high pH 10.0 and 11.8 reaction buffers

were dramatically superior to the 1.5X cloned *Pfu* buffer, further demonstrating the enhancing effects of high pH for PCR amplification with *Pfu*-Sso7d (figure 3).

To further demonstrate the enhancing effects of high pH on PCR amplification with the chimeric *Pfu*-Sso7d DNA polymerase, amplification of the 19kb human beta globin genomic target was compared using the *Pfu*-Sso7d / *Pfu* Turbo blends (0.25U *Pfu*-Sso7d + 2.5U or 5.0U *Pfu* Turbo) and 0.83U and 1.3U of *Pfu*-Sso7d in the pH 10.0 PCR reaction buffer with a 30 second per kb extension time (figure 4). The significant difference between these PCR reactions, since they all use the pH 10.0 buffer, is the amounts of *Pfu*-Sso7d in each reaction (i.e. 0.25U *Pfu*-Sso7d for the blends and 0.83U and 1.3U *Pfu*-Sso7d for the non-blend reactions). The reactions which have 0.83U and 1.3U *Pfu*-Sso7d without any cloned *Pfu* DNA polymerase (#3 and #4 figure 4) generated dramatically higher yields than the blend reactions (#1 and #2 figure 4) which only had 0.25U *Pfu*-Sso7d even though the total units of DNA polymerase were higher for the blend reactions (2.75U #1, 5.25U #2 for the blends and 0.83U #3 and 1.3U #4 for the *Pfu*-Sso7d reactions - figure 4).

PCR performance using a reaction buffer at pH 10.0.

The amplification efficiency of the 19kb human beta globin target with Herculase DNA polymerase, KOD hot start DNA polymerase and a unit titration of the *Pfu*-Sso7d chimeric DNA polymerase was compared (Figure 5). All enzymes were used in their optimal reaction buffers. The pH 10.0 buffer was used for *Pfu*-Sso7d, KOD hot start buffer for KOD hot start, and Herculase buffer for Herculase. 3% DMSO was added to the Herculase reactions which is optimal for the amplification of genomic targets over 10kb in length. A 30 second per kb extension time was used. Most PCR enzymes require an extension time of 1-2 minutes per kb for a target of this length. All unit amounts (0.25-1.3U) of *Pfu*-Sso7d in the pH 10 buffer generated PCR product. The Herculase and KOD hot start reactions did not generate any PCR product at this extension time.

The amplification of smaller genomic targets was also compared using the *Pfu*-Sso7d chimeric DNA polymerase in the high pH 10.0 PCR reaction buffer and KOD hot start DNA polymerase in KOD hot start PCR reaction buffer. A 900bp fragment of human alpha-1 anti trypsin (Hα1AT) was amplified with a 1 second total extension time using 1) 0.5U or 0.83U of *Pfu*-Sso7d in pH 10.0 or pH 11.8 PCR reaction buffers, and 2) 1U of KOD hot start in

KOD hot start PCR reaction buffer (figure 6). A 2.6kb fragment of Hα1AT was amplified with a 2 second per kb extension time (5 second total extension time) (figure 7) and a 30 second per kb extension time (1 minute 18 second total extension time) (figure 9) using 0.5U, 0.83U and 1.3U of *Pfu*-Sso7d in the pH 10.0 PCR reaction buffer and 1.25U and 2.5U of KOD hot start in KOD hot start PCR reaction buffer. A 6kb fragment of human beta globin was amplified with a 10 second per kb extension time (1 minute total extension time) (figure 8) using 0.5U, 0.83U and 1.3U of *Pfu*-Sso7d in the pH 10.0 PCR reaction buffer and 1.25U and 2.5U of KOD hot start in KOD hot start PCR reaction buffer. The extension times for all targets were shorter than the standard time for most PCR enzymes. 30 seconds to 2 minutes per kb is standard for most PCR enzymes. For all targets, the chimeric *Pfu*-Sso7d DNA polymerase in the high pH PCR reaction buffers displayed vastly superior performance at all unit amounts (0.25-1.3U per reaction).

By the use of a high pH PCR reaction buffer with a processive chimeric *Pfu* DNA polymerase (in the presence of PEF/dUTPase), PCR extension times were substantially reduced for the amplification of genomic targets. For genomic targets between 1-6kb an extension time of 1min/kb for a non-chimeric DNA polymerase/ DNA polymerase formulation was reduced to 1-10 seconds per kb. For genomic targets between 17-19kb an extension time of 2 min/kb for a non-chimeric DNA polymerase/ polymerase formulation was reduced to 30 sec/kb. The high pH reaction buffer / chimeric DNA polymerase / chimeric DNA polymerase blend combination is used in the same way as a conventional PCR reaction buffer / DNA polymerase/ DNA polymerase blend combination and can be used in any primer extension application, including PCR, to produce high product yields with shortened extension times. The main application would be for the amplification of genomic targets, which typically require extension times of 1-2 minutes per kb and can take hours to amplify. Extension times could be reduced to 1-30 seconds per kb, or shorter, with the high pH buffer and chimeric DNA polymerase. Amplification times could be dramatically reduced, substantially improving PCR applications. Other applications include RT-PCR, site-directed mutagensis and random mutagenesis. A high pH reaction buffer / chimera combination used in all of these applications would increase length capability and shorten reaction times and highly increase overall performance in all standard protocols.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, cell biology, microbiology and recombinant DNA techniques, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, 1989, Molecular Cloning: A Laboratory Manual, Second Edition ; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Nucleic Acid Hybridization (B.D. Harnes & S.J. Higgins, eds., 1984); A Practical Guide to Molecular Cloning (B. Perbal, 1984); (Harlow, E. and Lane, D.) Using Antibodies: A Laboratory Manual (1999) Cold Spring Harbor Laboratory Press; and a series, Methods in Enzymology (Academic Press, Inc.); Short Protocols In Molecular Biology, (Ausubel et al., ed., 1995).

All patents, patent applications, and published references cited herein are hereby incorporated by reference in their entirety. While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.